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APPLIED IMMUNOLOGY OF MINERAL OIL ADJUVANTS

Fred M. Davenport, M.D., Ann Arbor, Mich.

In 1956, in reviewing the historical development of the use of water-in-oil emulsions as immunologic adjuvants, Jules Freund wrote the following succinct statement. "The past of the whole subject is long, but its history is rather short. Indeed in retrospect one wonders at the disproportion between the length of time and the fewness of the steps that separate the basic observations from their successful application."[1] In the preceding year, Dr. Freund addressed the American Academy of Allergy at its Eleventh Annual Meeting and one of his characteristically cautious conclusions was, "The use of adjuvants may find application in prophylactic immunization and preseasonal treatment of allergic patients."[2]

Jules Freund was responsible for many of the steps which made it possible to bridge the gaps between the basic observations and their application.[3] Today, therefore, in recognition of the central role he played in the development of mineral oil adjuvants, it seems appropriate to recount before this society some of these basic observations and to interpret their significance, since, by now, the unique advantages of mineral oil adjuvants have been utilized successfully for prophylactic immunization of man and for the treatment of allergic patients.

From the Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan.

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MECHANISM OF ACTION OF MINERAL OIL ADJUVANTS

The results of studies on the mechanism of action of mineral oil adjuvants explain in part why they are so effective. It was shown early by Freund and associates that an adjuvant effect could be induced far more readily by the use of mineral oils than by the use of animal or vegetable oils. Furthermore, for success, it was found necessary to incorporate the antigen in a water-in-oil emulsion, coating it thereby with a non-metabolizable substance.[4, 5] These findings immediately suggested that one of the advantages of the mineral oil adjuvants was protection of the antigen against destruction by humoral factors or by cells incapable of engaging in antibody production. Studies on the distribution of mineral oil emulsions after inoculation have shown that a large part of the injected mass persists locally for many months. A portion, however, migrates very early to regional lymph nodes and, later, more may be found in remote lymph nodes or at other sites.[6-8] Thus, the emulsified inoculum appears to serve not only as a local protected depot of antigen, but as a source of emulsified antigen which slowly and progressively leaves the depot in a shielded state and reaches, through the lymphatic system, remote foci, where, it is believed, the emulsion breaks down and antibody production is stimulated in suitable cells.[9, 10] Probably the macrophages which accumulate about the antigen depot play an important role in the transporation of the emulsion droplets. The net result provides not only an effective primary but an efficient "built in booster" stimulus to antibody formation.[11-16]

An alternate view is that the site of inoculation constitutes an antibody forming "organelle," and that the cells which accumulate about the depot produce the major portion of the circulating antibody.[17-21] However, there are data that make this explanation of the mechanism of action of mineral oil adjuvants difficult to support.[16, 22, 23]

The suggestion has also been made that the mineral oil component of the emulsion causes a hypertrophy and hyperplasia of cells capable of forming antibody and thereby prepares them to function more effectively upon the arrival of antigen.[24, 25] The conditions of the experiments in animals which support this hypothesis are so unlike those to be reported today that the likelihood of such a factor operating in man seems not very great. More certain is the conclusion that the mode of action of mineral oil adjuvants is complex and as yet has not been fully elucidated.

EARLY EXPERIMENTS IN MAN

Following the demonstration by Friedewald[26] that an emulsified influenza virus vaccine yielded higher antibody levels and greater protection in mice than did an aqueous vaccine, W. Henle and G. Henle[27] began a study in 1944 on antibody response of human beings to mineral oil adjuvant influenza virus vaccine. Their vaccine was compounded by emulsifying one volume of virus concentrate in five volumes of a mixture consisting of one part emulsifier* and four parts of mineral oil.† Eighty subjects were given

*FALBA—distributed by Pfalz and Bauer, Inc., New York.
†Atreol No. 9 of the Atlantic Refining Company.

0.3 ml. of this vaccine subcutaneously. For comparison, an additional eighty subjects were given aqueous vaccine containing the same amount of virus. Geometric mean hemagglutination inhibiting antibody titers found against strains of influenza A and B in sera obtained from both groups of subjects at various intervals are reproduced in Fig. 1.

Here, two of the immunologic advantages of mineral oil adjuvants are clearly seen. Note that antibody titers induced by adjuvant vaccine are markedly higher and persist at high levels far longer than those obtained with aqueous vaccine. However, this particular adjuvant vaccine was not well tolerated by the subjects. Nodules up to 3 cm. in diameter could be palpated in the subcutaneous tissues of most of the individuals. These decreased in size but were still evident in 40 per cent of the cases six months after vaccination. Two subjects developed abscesses at the site of inoculation about two months after the injection. Fortunately, Freund[28] found that the use of Arlacel A* as emulsifier and a light mineral oil as vehicle tended to prevent the occurrence of such abscesses in animals.

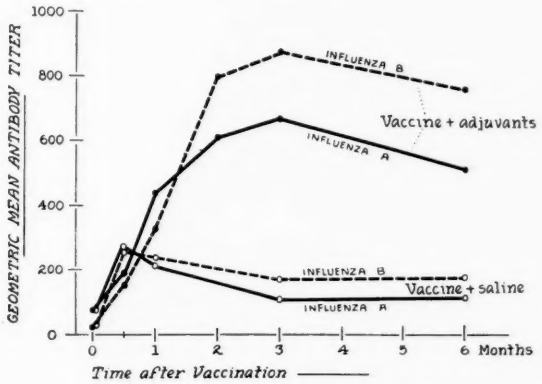

Fig. 1.—Geometric mean antibody titers (initial serum dilutions). (From Henle and Henle, Proc. Soc. Exper. Biol. & Med. 59: 179, 1945.)

In the course of studies in monkeys, first on antibody response to polio viruses and later to strains of influenza, Salk and colleagues[29, 30] called attention to two additional factors that minimize the risk of encountering undesirable late local reactions. They are: the employment of an antigen that, by itself, does not induce a significant inflammatory response, and the use of the intramuscular rather than the subcutaneous route of inoculation. Gross examination of tissues of animals receiving injections of emulsions that met these four criteria showed no evidence of acute inflammation. The mechanical effects of muscular action distributed the emulsion in fine droplets over wide areas, particularly along muscle fibers. The droplets of emulsion were free and not bound by exudate. Microscopically dense aggregates of mononuclear cells were seen. In contrast, when the emulsion was given subcutaneously, a thin-walled cyst was formed containing the bulk of the inoculum, surrounded by a capsule of mononuclear cells.[30]

*Mannide monoleate—The Atlas Powder Company, Wilmington, Del.

As pointed out by Francis[31] and by Salk[32] in 1953, early concerns were the possibility of a carcinogenic effect of the light mineral oil and the risk of sensitization. Careful study of the data on the carcinogenicity of mineral oils was begun before the first injections were given to human beings and have been continued to the present. The accumulative information shows that the oils of low viscosity employed in these studies are free of the physical and chemical characteristics associated with agents that are carcinogenic. The development of experimental hypersensitivity syndromes (for example, allergic encephalitis) is, as Freund[2] had shown, primarily dependent upon

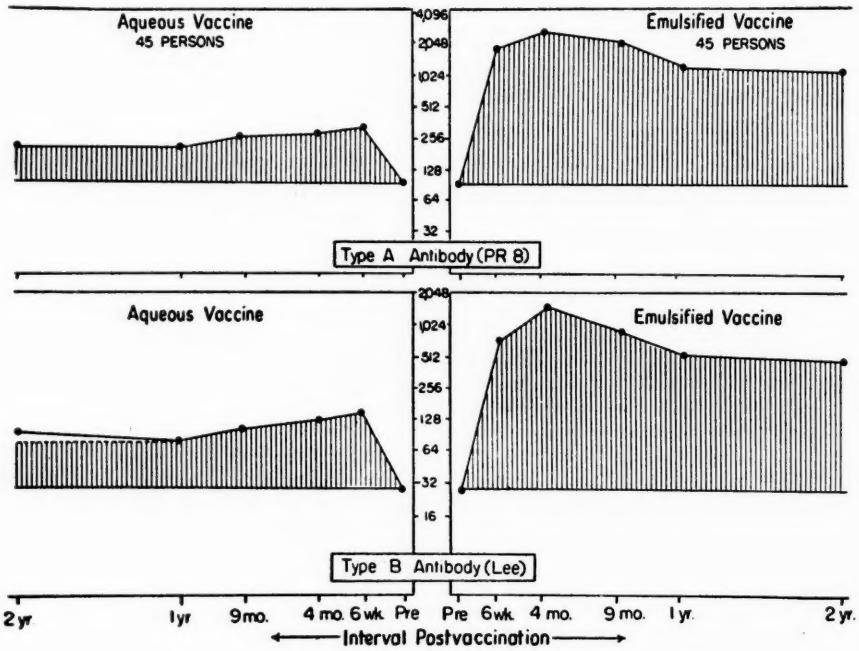

Fig. 2.—From Salk et al., J. A. M. A. 151: 1169, 1953.

adding tubercle bacilli to the mixture. Of course, these were omitted from the preparations intended for use in man, and to date no evidence has been found that the use of mineral oil adjuvants induces allergic symptoms in human beings.

From this base, the studies were systematically extended in man. Fig. 2 illustrates the results obtained by Salk and associates[32] when small groups of persons were given 300 chicken cell agglutination (CCA) units of a multistrain vaccine in either aqueous or emulsified form. By immediate local reactions to the vaccines, the two groups could not be distinguished.[33] By antibody response they were quite different. Again the higher and more persistent antibody levels that follow inoculation of emulsified vaccine are clearly evident. In this study it was noted that the slight drop in geometric mean antibody titers found two years after inoculation with adjuvant vaccine was not due to a decline in the antibody levels of all subjects. Rather the

drop was due to a fall in the concentration of antibody in the sera of those individuals who showed the highest initial response to vaccination. Davis and associates[34] have observed similar phenomena in a study with a serologic follow-up of three years.

Fig. 3 illustrates another, and a very important, advantage of mineral oil adjuvants. By their use a great economy can be effected in the amount of antigen needed to achieve a given level of antibody. Salk and his co-workers[32] compared the fold increase in geometric mean antibody titers in groups of persons given various amounts of influenza virus in aqueous suspension or emulsified in oil. The data demonstrate that antibody response to emulsified vaccines is much more effective than is the response to aqueous vaccines of the same virus content. Note that giving one CCA unit of virus in adjuvant produced essentially the same increase in antibody as did giving

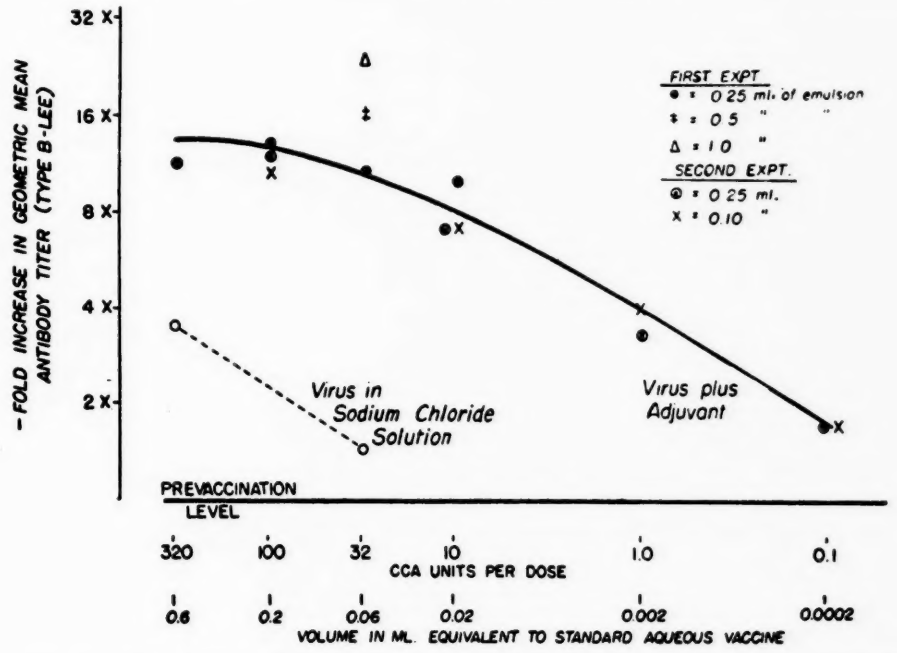

Fig. 3.—Antibody response to diminishing quantities of influenza virus vaccine emulsified with mineral oil or in saline. Vaccine contained equal parts of PR 8, Cuppett and Lee strain and was diluted before emulsification. (Groups of approximately 50 young adults.) (From Salk et al., J. A. M. A. 151: 1169, 1953.)

320 CCA units in saline. Above a dose of 32 CCA units of virus per strain in 0.25 ml. adjuvant, the yield of antibody was scarcely greater even when the amount of antigen given was increased tenfold. However, the yield of antibody was enhanced progressively when 32 units of virus were incorporated in 0.5 or in 1.0 c.c. of emulsion. Perhaps the larger volume is more effective because it becomes dispersed more readily. Yet, since adequate levels of antibody can be achieved with the smaller volumes, the size of the inoculum used in subsequent studies was maintained at 0.25 ml.

EXTENSION OF THE STUDIES IN MAN

With this and other experience,[35] the trials were extended by Salk and his group[32] at Fort Dix, New Jersey, in the winter of 1951-1952. Despite the fact that no late cystic swellings were encountered in the first 2,000 persons vaccinated with the preparations under test, seventeen subcutaneous fluctuant cystic swellings were observed among 6,000 persons inoculated with a polyvalent influenza virus vaccine in the fall of 1951. In experiments begun in the same year, Bell observed nineteen such swellings after vaccination of 2,426 individuals with monovalent vaccines. As Bell's population was followed it became apparent that cyst formation might occur many months after the vaccine was given.[36] In both experiences, the greater frequency of cyst development in Negroes was noted. The results of further inquiry by Salk and Bell revealed that the cysts occurred in persons given lots of vaccine containing a particular batch of Arlacel A. Salk concluded from extensive experiments in animals that the untoward reactions encountered in man could be attributed to the presence of impurities in Arlacel A which could be removed by further refinement of the product without loss of emulsifying activity. He developed a "toxicity" test in mice and guinea pigs for determining the presence of harmful materials in different batches of Arlacel A, and this test has since been used as a guide for selection of lots of Arlacel A intended for use in vaccine prepared commercially for additional field trials by the Commission on Influenza. Beginning in 1952, the more highly refined mineral oil, now designated Drakeol 6VR,* was substituted for Bayol F.† To date, over 100,000 doses of adjuvant influenza virus vaccine have been given in those trials and the results have been most gratifying. The vaccine formula most frequently used is five parts virus concentrate, one-half part purified Arlacel A, and four and one-half parts Drakeol 6VR. In our experience, only an occasional cyst has been encountered since the refined and pretested Arlacel A and Drakeol have been employed. The recorded rates of cyst formation vary from three in 10,000 inoculations to twenty-three in 10,000 injections. The higher rate was found in children less than 6 years of age. Vaccines made with influenza Type B strains[36] or with mouse adapted rather than egg passaged Type A strains[37] are said to yield higher rates of late local reactions. However, the data are too limited to establish that strain differences play a significant role. At Fort Ord, Fort Dix, Great Lakes Naval Training Center, and at Lowry Air Force Base, where most of the adjuvant vaccine field trials of the Commission on Influenza have been carried out, the occurrence of cysts has not been reported. One previously unrecorded case was encountered at Sampson Air Force Base in 1955.[38] Since military populations are not stable ones and because the late local reactions may present as long as twenty-seven months after inoculation,[36] a two year survey was made by the Division of Preventive Medicine of the Office of the Surgeon General, of records covering surgical procedures carried out in military hospitals. No evidence was found that cysts requiring drainage were occurring as

*Pennsylvania Refining Co., Butler, Pennsylvania.
†Esso Co., New York City.

a result of vaccination with mineral oil adjuvants.[39] Thus, the evidence accumulated from these efforts in the last ten years clearly demonstrates that late local reactions can be avoided or minimized to an acceptable level if proper materials are used correctly. To complete the record, a systematic and comprehensive follow-up is now underway on a sample of the large group given adjuvant vaccine a decade ago in order to be as certain as possible that undesirable sequelae of the use of adjuvants have not occurred at a low frequence and thereby escaped attention.

TABLE I. RESULTS OF FIELD TRIALS OF THE COMMISSION ON INFLUENZA WITH ADJUVANT INFLUENZA VIRUS VACCINE

INVESTIGATOR	EPIDEMIC STRAIN	YEAR	PROTECTION RATIO
Davenport et al.[40]	B-prime	1954	2.7
Lennette[41]	A-prime	1957	5.1
Lennette[41]	Asian	1958	7.1
Meiklejohn[42]	Asian	1960	16.7

To warrant adoption of adjuvant preparations for general use, proof is needed not only of safety but also of efficacy. Table I summarizes the results of the adjuvant vaccine field trials carried out in military recruits under the auspices of the Commission on Influenza. Protection is expressed as the ratio of the attack rate observed in placebo inoculated controls to that of vaccinated subjects. In 1954-1955 a protection ratio of 2.7 was measured using a vaccine containing the Lee strain of 1940.[40] In the same year, aqueous vaccines were found to be ineffective, owing to the fact that the epidemic strains then prevalent were quite different antigenically from the Lee strain. That result illustrates still another advantage of adjuvant vaccines, that is, a broadening of antibody response due to enhanced reaction to minor antigenic components of the strain given.[32] In the spring of 1957, Lennette at Fort Ord observed a protection ratio of 5.1 against influenza A prime. In the next year, Lennette also observed a protection ratio of 7.1 against Asian influenza.[41] The adjuvant vaccine tested contained only 150 CCA units of Asian virus, while the aqueous preparation recommended for use in civilians at that time contained 400 CCA units of the same strain. This was a striking demonstration that adjuvant vaccines can induce an important degree of protection against strains that are so novel antigenically that the vaccination constitutes the primary exposure. In 1960 at Lowry Air Force Base, Meiklejohn observed a protection ratio of 16.7 against Asian influenza. The vaccine under test contained 100 CCA units of an Asian strain and 50 CCA units each of five other strains of influenza virus. In addition it contained one half the standard amount of type 4 and 7 adenoviruses. In the same population, that vaccine yielded a protection ratio of 10 to 1 against ARD. The results of Meiklejohn's study provide a clear demonstration of the practicality of incorporating a number of different viruses in a single adjuvant preparation.[42]

From these data, it is evident that Dr. Freund's expectation that mineral oil adjuvants might prove useful in prophylactic immunization has been realized.

APPLICATIONS IN ALLERGIC SUBJECTS

While to date, the data are less extensive on the use of adjuvants in preseasonal treatment of allergic subjects, it seems likely that Freund's concept that they may prove useful for that purpose has also been realized.

Loveless[43] in 1947 began a long-term study of 117 patients who received alternate courses of customary treatment with aqueous extracts and with extracts emulsified in mineral oil. Ten years later she summarized her observations. Clinical and statistical comparisons were made between depot therapy and conventional multivisit treatment in ragweed hay fever. Each subject served as his own control and in the last few years of the study, placebo emulsions were given to a portion of the group to permit an evaluation of psychic and other non-immunologic influences on the clinical results. As Loveless points out, clinical and statistical analysis of the results obtained presents many difficulties[43] and, because there is as yet no general agreement on the criteria to be used for evaluation, direct comparisons of the findings of different investigators become practically impossible. Therefore, in this presentation no attempt will be made to discuss the quantitative aspects of the various reports cited; rather the authors' general conclusions will be presented. Loveless decided that preseasonal therapy of ragweed hay fever with an adjuvant depot inoculation provided essentially the same clinical and immunological benefits as did a series of inoculations with aqueous extracts. While not reported in detail, Loveless stated that the findings in patients with ragweed hay fever were duplicated in 200 additional patients treated in a like manner for allergies to other pollens, house dust, or animal danders.[43]

Later, Brown became interested in the application of preseasonal therapy with extracts of allergens emulsified in mineral oil and has recorded his observations in a number of publications. As of June 1960, 1,064 patients with ragweed pollenosis had been treated by means of a single annual injection of emulsified extract. The results were apparently satisfactory. Clinical responses of patients treated with adjuvant were compared with those of approximately 500 patients who received traditional types of treatment. Only about 14 per cent of the patients given a single injection of emulsion reported symptoms, while almost 40 per cent of the conventionally treated group were symptomatic.[44] Brown has also reported success in treating 564 patients allergic to grass pollen[45] and in treating 379 persons sensitive to tree pollen.[46] In commenting on an earlier study of 383 patients with grass pollenosis, Brown confirmed Loveless's conclusion that the results of emulsion therapy "were equal, if not superior to those achieved by conventional treatment with extracts prepared in saline."[47] Beneficial results from the use of adjuvants in the management of hypersensitive persons has likewise been reported by a number of other investigators, including Mansmann,[48] Cooke and colleagues,[49] Furstenberg,[50] and Feinberg and co-workers.[51]

In the course of treating 508 patients allergic to house dust, Brown encountered sixty-five patients who developed cysts at the site of inoculation. By reducing the amount of house dust given, by refining the extract, and by

injecting the emulsion intramuscularly, the development of cysts was prevented. It was Brown's opinion that the patients benefited by the treatment with emulsified house dust and that, in those who developed cysts, the clinical results were long lasting.[52]

Cyst formation at the site of inoculation was also encountered by Loveless. She stated that the disadvantage of the unduly persistent nodule was chiefly cosmetic.[43] Perhaps the nodule provides a daily reminder that the allergist's therapy is always with the patient, and this may account in part for the placebo effect she described.

Yet these untoward reactions are undesirable and apparently can be prevented. As a result of the total experience with prophylactic immunization and treatment of allergy it is recommended that the volume of the inoculum be kept as small as practicable, that it be given intramuscularly if feasible, that it be compounded with highly refined reagents, pretested where possible for toxicity or inflammation producing potential, and that special care and caution be used in administering mineral oil adjuvants to children and to Negroes of all ages.

DEVELOPMENTS THAT SUGGEST NEW APPROACHES

Objections have been raised to the application of two of these principles to the treatment of allergic subjects. Here the trend has been to incorporate in the emulsion many more units of extract than can be tolerated by patients if they were to receive them in a single aqueous dose. The concept has been to build up as large a depot as one can with safety, in order to provide in a single dose enough extract to tide the patient over the period of exposure. This objective explains the frequent use of the words depot or repository therapy of allergies and accounts for increases in the volume of emulsion injected. Because the dose is large, because an early release of a portion of the emulsified extract occurs after inoculation, and, because absorption from inoculations given intramuscularly may be more rapid than from subcutaneous sites, the tendency has been to give the larger volumes of emulsified extracts subcutaneously in order to minimize the risk of serious systemic or generalized reactions.

While it is hazardous to transfer directly knowledge gained from experience in the field of prophylactic immunization to treatment of hypersensitive patients, the results of two studies carried out in our laboratory in Ann Arbor suggest possible ways of successfully treating allergic subjects by giving a small dose intramuscularly.

In a careful study of the physical properties of emulsified influenza virus vaccines and the adjuvant response, Berlin[23] found it possible to vary the viscosity of emulsified vaccines by using petroleum oils of different viscosities, by varying the oil to water ratio, by varying the time of agitation used to make an emulsion, or by adding Thixcin,* a commercially available lyophylic powder.

*Specially prepared 12-hydroxystearin from the Baker Castor Oil Co., Bayonne, N. J.

Fig. 4 reproduces a portion of the data obtained on the relation between antibody response and viscosity of the adjuvant vaccine tested. It is evident that vaccines of high viscosity induced low antibody levels, while vaccines of low viscosity yielded high antibody levels. One interpretation of these data is that the higher the viscosity of the emulsion, the more slowly the aqueous phase is released after injection.[23, 53] Hence it would seem feasible to control the rate of release of pollen or other extracts by controlling the physical characteristics of the emulsion used.

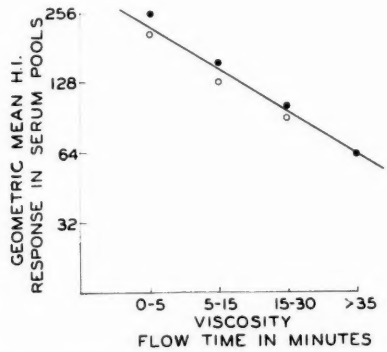

Fig. 4.—Inverse relation between antibody response and viscosity.

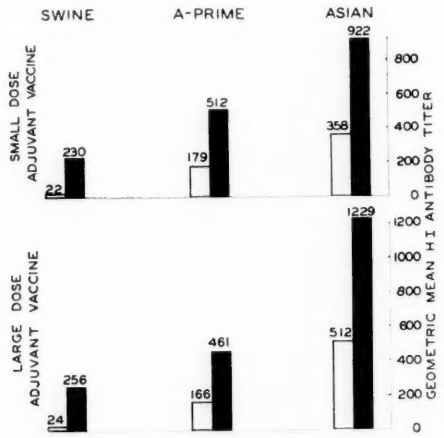

Fig. 5.—Antibody response of children to two large injections of small or large dose adjuvant vaccine.

From the results of studies on antibody response to small doses of adjuvant influenza virus vaccine, the hypothesis can be suggested that perhaps it is unnecessary to give the high doses currently used in repository therapy of allergy. While it has been shown that as little as 10 CCA units of influenza virus given in adjuvant will induce high antibody levels when used for "booster" stimulation,[32] it is also true that large doses of virus in adjuvant yield but low antibody levels when used for primary stimulation.[54] However, if an adjuvant vaccine is used to provide both the primary and the secondary stimulus, in the form of two doses separated by an interval of three months,

high antibody levels can be achieved with small amounts of virus even to strains that the subject is being exposed to for the first time in this schedule of vaccinations.[55]

Fig. 5 illustrates the remarkable efficiency of mineral oil adjuvants when used in this way. One group of children was given a vaccine containing 10 CCA units of each of the strains tabulated and three months later was reinoculated with the same vaccine. In like manner, a second group received two doses of adjuvant vaccine containing 50 CCA units each of swine and A-prime strains and 100 CCA units of Asian virus. Serum samples were obtained at the time of the second vaccination and six weeks later. The geometric mean antibody titers shown in Fig. 5 represent antibody levels found after the first and second vaccination.

Note that, after the first injection, antibody levels in both groups against swine virus are low whereas against A-prime and Asian viruses they are considerably higher. For these children, swine virus is a primary antigenic experience, whereas they have had prior exposure to A-prime and Asian influenza. After the second dose, adequate protective levels of antibody against all strains of virus were present. It is especially pertinent to observe that the levels achieved after two 10 CCA unit doses are approximately equal to those found after two 50 or 100 CCA unit doses.[55]

If these findings on emulsion viscosity and on dose of antigen can be applied to the treatment of allergic subjects, the possibility exists that even highly sensitive patients can be treated safely by using small volumes of emulsion given intramuscularly at appropriate intervals without incurring immediate systemic reactions or delayed cyst formation. Two office visits per annum rather than one would be a small price to pay for these advantages.

CONCLUSION

Clearly all of the problems inherent in applying mineral oil adjuvants to prophylactic immunization or preseasonal treatment of allergy have not been solved. Yet important advances have been made since Jules Freund spoke before this society six years ago. The way now seems open to extend these observations in man carefully, cautiously, and objectively, so as to reap the full benefits of the basic work of Freund and of those who have likewise built upon that foundation of primary observations and concepts.

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EFFECTS OF WATER-SOLUBLE ADRENOCORTICAL STEROIDS ON PASSIVE CUTANEOUS ANAPHYLAXIS OF THE GUINEA PIG

Richard E. Kauff, New York, N. Y.

THAT CORTISONE has no effect on passive cutaneous anaphylaxis was shown in 1952[1] and these observations were confirmed in 1956[2] using threshold amounts of antibody. Later, however, Prochazka-Fisher and Cooke[3] have reported that the administration of water-soluble hydrocortisone intravascularly to guinea pigs in which passive cutaneous anaphylaxis is elicited has resulted in a reduction of the intensity of the reaction and an increase in the threshold concentration of antibody required for effective reaction. Osler and his coworkers[4] have emphasized that, in order to demonstrate inhibition of the passive cutaneous anaphylactic reaction, one must employ not only a threshold amount of antibody, but also that corresponding minimal amounts of antigen should be used to elicit the reaction. Recently, moreover, more potent adrenocortical steroid preparations have become available, and it, therefore, seemed important to re-evaluate the effect of steroids on passive cutaneous anaphylaxis in the guinea pig using these preparations and threshold levels of reactants.

MATERIALS AND METHODS

a. *Antibody.*—Rabbit antibovine plasma gamma-globulin (RaaBGG) containing 48.0 μg antibody N per milliliter was used. The concentrations employed are given in Table I.

b. *Antigen.*—Bovine plasma gamma-globulin, fraction II (BGG),* was diluted to give a final concentration of 15 μg protein N per milliliter.

c. *Histamine.*—A solution of histamine containing 10 μg per milliliter was employed.†

d. *Compound 48/80.*—A solution of Compound 48/80‡ was used which contained 40 μg per milliliter.

e. *Steroids.*—The steroids used in this study were hydrocortisone hemisuccinate and methylprednisolone hemisuccinate.§

From the Department of Pathology, New York University, School of Medicine, 550 First Ave., New York 16, N. Y.

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*From the Armour Pharmaceutical Company, Kankakee, Ill. (lot # R30005).

†Histamine dihydrochloride, Fisher.

‡From the Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.

§Supplied as Solu-Cortef and Solu-Medrol, respectively, by the Upjohn Company, Kalamazoo, Mich.

f. *Dye.*—Evans blue dye was used as a 1 per cent solution in 0.15 M NaCl.

g. *Diluent.*—All materials requiring further dilution were prepared with 0.15 M NaCl.

h. *Animals.*—Albino guinea pigs (Hartley strain), weighing approximately 250 grams, were used in this study without regard to sex distribution.

The technique of passive cutaneous anaphylaxis (PCA) as described previously by Ovary[5, 6] was used in this study. All animals received intradermal injections of antibody (RaaBGG), histamine, and Compound 48/80 parasagittally on carefully shaved areas of the back (each of the two latter compounds were injected immediately before challenge), each solution injected in a constant volume of 0.1 ml. Histamine and Compound 48/80 were included in this experiment as controls in order to study the effects of these steroids on the permeability of the minute blood vessels and on histamine liberation, respectively. A three-hour latent period was provided to allow fixation of the antibody, after which time 1.0 ml. challenging doses of an antigen-dye-steroid mixture (antigen-dye-saline in control animals) were administered intravenously through the veins of the foot.

At the time of intradermal preparation, animals in the experimental series received intraperitoneal injections of steroid: 50 mg. of hydrocortisone hemisuccinate, or 10 mg. of methylprednisolone hemisuccinate, in a volume of 1 ml. Control animals received 1.0 ml. of physiologic saline by the same route.

Preliminary studies with the RaaBGG showed that less than 0.08 μg antibody N per intradermal site gave poor reactions after a challenging dose of 15 μg antigen N. On the other hand, 0.08 to 0.12 μg antibody N per intradermal site gave poor reactions when less antigen was used for challenge. It thus seemed reasonable to employ 15 μg antigen N per guinea pig in the challenging intravenous injection. At the time of challenge, each control animal received a mixture of 0.5 ml. dye and 15 μg antigen N in 0.5 ml., and each experimental animal received 15 μg BGG N, 0.5 ml. dye, and either 25 mg. of the hydrocortisone preparation or 10 mg. of the methylprednisolone preparation in a total volume of 1.0 ml. per guinea pig.

All animals were killed and skinned fifteen minutes after the challenging injection. Reactions were measured on the internal surface of the skin.

RESULTS

Results are given in terms of the diameter of the colored spot in millimeters.[6] The time after elicitation of the reaction did not appear to be a factor, both the control and experimental groups showing the appearance of the reaction to the same extent at the same time after challenge. The data in Table I show a representative experiment.

DISCUSSION

The results given in Table I show that the use of intravenously administered water-soluble adrenocortical steroids do not significantly alter the size of the cutaneous reaction elicited at threshold concentrations of antibody and antigen

TABLE I

INTRAVENOUS DOSE	ANIMAL NUMBER	INTRADERMAL INJECTIONS (DIAMETER OF REACTION IN MM.) RaaBGG N/0.1 0.12	0.08	0.02	HISTAMINE 1 μg	COMPOUND 48/80 4 μg
Control Series	1	20	20	nd	20	25
15 μg BGG N and dye	2	10	0	nd	20	25
	3	20	18	nd	18	25
	4	15	13	nd	20	22
	5	12	tr	nd	20	18
	6	16	15	nd	18	16
	7	15	12	nd	16	16
	8	15	14	nd	18	16
	9	nd	17	tr	22	17
	10	nd	17	tr	18	17
	11	nd	16	tr	18	16
	12	nd	15	0	20	15
	13	nd	13	0	17	17
	14	nd	15	0	18	15
	15	nd	16	0	20	17
	16	nd	15	tr	20	14
Average		14.1	13.5	NC	18.3	18.4
Experimental Series A	17	20	20	nd	20	25
15 μg BGG N, dye, and 25 mg. hydrocortisone	18	10	0	nd	20	25
	19	20	18	nd	18	20
	20	13	13	nd	20	22
	21	10	10	nd	14	8
	22	13	0	nd	15	12
	23	0	0	nd	16	15
	24	15	13	nd	20	18
Average		12.6	11.7	nd	19.1	18.1
Experimental Series B	25	0	0	nd	20	20
15 μg BGG N, dye, and 10 mg. methylprednisolone	26	16	20	nd	15	25
	27	15	13	nd	15	20
	28	13	10	nd	15	20
	29	13	tr	nd	16	16
	30	16	16	nd	15	15
	31	0	0	nd	12	16
	32	16	16	nd	16	16
	33	nd	20	tr	17	15
	34	nd	15	0	16	15
	35	nd	17	tr	20	17
	36	nd	16	0	17	16
	37	nd	23	17	20	15
	38	nd	0	0	18	17
	39	nd	20	0	19	17
	40	nd	15	0	15	14
Average		11.1	12.3	NC	16.7	17.1

Note: BGG = bovine gamma globulin; RaaBGG = rabbit antibovine gamma globulin; nd = not done; NC = not calculated.

by PCA in the guinea pig; moreover, these drugs do not alter the temporal development of the reaction after challenge, the permeability of the minute blood vessels under the influence of locally injected histamine, or the release of histamine by a well-known histamine liberator (Compound 48/80). The slight alterations in reactivity observed in this study in the experimental animal series were of no significance and were within the limits of differential reactivity observed in the control animal series. These variations are expected even in an animal population comprised of the same strain and weight.

It has been established that ACTH and corticoids do not prevent the union of antibody and antigen in the guinea pig, nor do these agents alter the subsequent effects of such a union.[7] Furthermore, other workers have demonstrated that reactions leading to the release of active histamine in vivo in the guinea pig are not impeded by steroid preparations. Reactions investigated include active anaphylaxis,[8-11] passive anaphylaxis,[8] and exogenous histamine intoxication administered by aerosol.[11] Dragstedt, Mills, and Mead,[12] Carryer and Code,[13] and Spain, Fontana, and Strauss[14] have each used the appearance of histamine in biologic and test-tube systems as an indication of antigen-antibody reaction and have found that the appearance and final concentration of histamine and histamine-activity is unrelated to the steroid concentration of their respective systems. Kass and Finland[15] have recently reviewed the action of steroids and have concluded that they have no effect on those cutaneous reactions which develop rapidly and are associated with the release of histamine or histamine-like substances.

Germuth, Ottinger, and Oyama[16] have shown that steroids offer more protection to rabbits than guinea pigs against the Arthus reaction, but have confirmed that steroids do not protect the guinea pig against active or passive anaphylactic shock. The reasons for the decreased Arthus reaction in cortisone-treated guinea pigs are not known.

Prochazka-Fisher and Cooke[3] have reported finding an appreciable inhibition of PCA in the guinea pig after the administration of one dose of water-soluble hydrocortisone at the time of intravenous challenge. The importance of using minimal amounts of antigen was recently stressed as essential for inhibition studies on PCA.[17] It has been established that cortisone, when given in doses and for periods of time greater than those required to modify delayed hypersensitivity of the tuberculin type, does not effect PCA, even when threshold amounts of antibody are used.[2] Using more potent adrenocortical steroid preparations in high, repeated dosage with the sensitive technique of minimal antigen doses, we did not observe any inhibition under the experimental conditions described.

SUMMARY

Hydrocortisone and methylprednisolone, both as the water-soluble hemisuccinate, have no effect on the permeability of the minute blood vessels produced by histamine, histamine release by Compound 48/80, and do not inhibit the passive cutaneous anaphylactic reaction in the guinea pig when administered at the time of passive sensitization and again three hours later at the time of intravenous challenge.

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A ONE-YEAR AEROALLERGEN SURVEY OF PUERTO RICO

Correlation of Results With Clinical Findings in 300 Patients

Eduardo R. Pons, Jr., M.D., and Maria E. Belaval, M.S., San Juan, Puerto Rico*

THE INCREASE in business and pleasure travel to Puerto Rico, as well as the large migration of Puerto Ricans to the United States, has aroused interest among practicing allergists on the mainland in the specific regional factors that may affect allergic patients in this island. With the exception of isolated air-borne fungus cultures,[1] of mention of possible fungus and bacterial allergens in Puerto Rico,[2] and of listings of potential hay fever plants,[3] no studies of the air-borne allergens of the island have been performed. This communication will present the results of the first one-year fungus and pollen survey of Puerto Rico; a partial report of this study has already been published.[4] The findings will be correlated with the clinical features of 300 patients with respiratory allergy studied in the island.

Puerto Rico is located in the West Indies at latitude 18° 30′ N, longitude 65° 35′ W. It is densely populated by 2,210,703 inhabitants (1950 census) in an area of only 3,421 square miles. The topography is varied, with a central axis of mountains surrounded by coastal plains. The tropical-marine climate is pleasantly influenced by trade winds; the yearly extremes of temperature in San Juan range beween 62° to 94°. Rainfall is 61.19 inches yearly and it is fairly well distributed throughout the year, although much the greater amount occurs from May to December, inclusive; the sun shines on an average of 360 days a year. Sugar cane has traditionally been the main agricultural crop but, in the last decade, industrial and touristic development has caused a distinct rise in living standards.

MATERIAL AND METHODS

The survey was carried out from Feb. 1, 1956, to Jan. 31, 1957, according to the standards of the American Academy of Allergy for air-borne allergen surveys in the United States.[5] Slides coated with petroleum jelly were exposed daily for twenty-four hours in a Durham air sampling apparatus located on the roof of a one-story building at San Patricio Hospital in suburban San Juan. Pollen grains and fungus spores were counted on the slides over an area

From the Medical Service and the General Research Laboratory, San Patricio Veterans Administration Hospital, San Juan, Puerto Rico.

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*Present address: 929 Park Ave., New York City, N. Y.

of 4.48 sq. cm. and the counts were reduced to an area of 1 sq. cm. Individual Hormodendrum spores were counted as recommended by Durham.[6] Simultaneous twelve-minute weekly exposures of Petri plates containing modified Mehrlich's medium were performed in three different locations in San Juan, namely, a third-floor balcony overlooking the ocean in a residential section, a third-story roof in the business district, and at San Patricio Hospital. The plates were kept at room temperature for five days, at which time the fungus colonies were counted. The colonies from the plates exposed at the Hospital were subcultured on Sabouraud's, Czapek, corn meal, and V-8 juice media, and morphological identification of the genera of fungi was carried out. The nomenclature of genera as established by Clements and Shear[7] was followed throughout.

The case records of 300 patients with bronchial asthma, allergic rhinitis, or both, were studied in regard to occurrence of seasonal symptoms, variation of symptoms during residence in Puerto Rico and in temperate zones, and skin test reactions by the scratch and intracutaneous methods to approximately 80 food and inhalant allergens. All the patients were studied by the senior author between 1952 and 1956. The selection of cases was limited to patients who had had symptoms of at least one year's duration and who showed definite clinical and skin test evidence of allergy to environmental factors. Patients with bronchial asthma who showed predominantly intrinsic factors were excluded from the study, as well as patients who showed inconclusive skin test reactions. The series is believed to be representative of cases seen in the practice of clinical allergy in Puerto Rico.

SURVEY RESULTS

The daily slide counts showed a marked predominance of fungus spores over pollen grains (Table I and Fig. 1). The range of the daily spore count was 2 to 2,193 per square centimeter, and the daily pollen counts varied from 0 to 15 per square centimeter. The average daily spore count for the entire year studied was 41.2 spores per square centimeter; that of pollen was only 2.2 grains per square centimeter. Although there were marked day-to-day variations in both fungus and pollen counts, no significant seasonal variations occurred. The high fungus count for December reflects one daily count of 2,193 spores per square centimeter, while the next highest spore count in the entire year was 163 per square centimeter.

TABLE I. AVERAGE DAILY COUNT OF FUNGI AND POLLEN (1956-1957) PER SQUARE CENTIMETER

	FEB.	MAR.	APR.	MAY	JUNE	JULY	AUG.	SEPT.	OCT.	NOV.	DEC.	JAN.
Fungi	25.9	33.9	28.7	38.8	36.9	34.3	36.7	38.1	42.3	37.2	114.8	32.6
Pollen	1.6	2.6	3.3	2.9	3.1	1.9	2.9	1.9	1.9	1.3	1.3	1.3

Upon comparing the monthly total of fungus spore counts per square centimeter with the Alternaria spore counts included in the Statistical Report of the Mold and Pollen Committee of the American Academy of Allergy for

1956,[7] we find that our counts are much higher than those reported for twenty-two states and Canada. There are only two exceptions: San Antonio (Texas) reported a total count of 1,294 Alternaria spores per square centimeter for the month of June, and Lexington (Kentucky) reported a count of 1,281 per square centimeter for the month of July. Both of these counts are higher than ours for the corresponding months, even though they include only Alternaria spores.

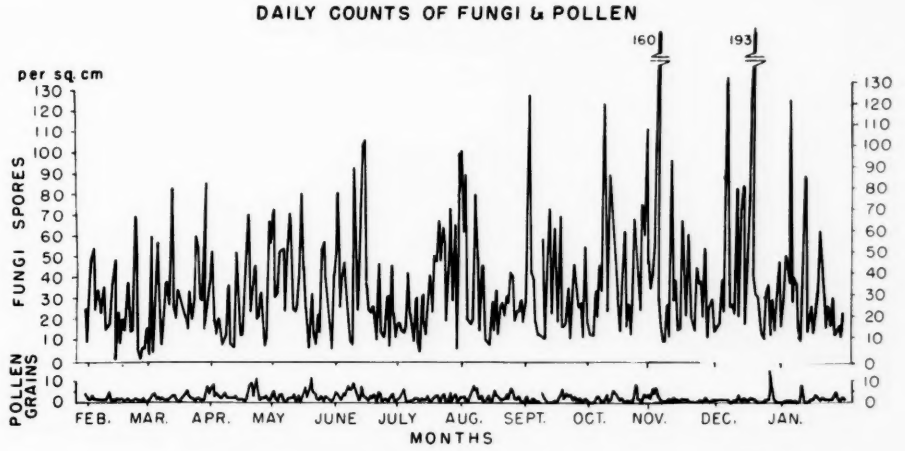

Fig. 1.

The total daily, weekly, and monthly fungus spore counts were analyzed in relation to corresponding climatological data of rainfall, temperature, dew point, wind velocity, and wind direction, as obtained from monthly reports of the U. S. Department of Commerce Weather Bureau. No obvious relationships were apparent. Simple correlation analyses were carried out with the daily spore counts throughout the year and relative humidity, and, independently, with the spore counts and dew point. No correlation was found to exist. It must be pointed out that the climatological data was obtained from readings taken at the Isla Verde Airport, at a distance of 5.6 miles from the slide exposure site.

TABLE II. WEEKLY AVERAGE OF PLATE FUNGUS COUNTS PER MONTH (1956-1957)

	FEB.	MAR.	APR.	MAY	JUNE	JULY	AUG.	SEPT.	OCT.	NOV.	DEC.	JAN.
Suburban area	48.5	41.4	58.2	22	39	31.7	117.6	27.5	122	12	49	64
Business area	12.2	10	14	20	8.2	9	9	13.2	46.5	14	13.5	8.7
Seashore	5.2	12.2	6.3	13	3.2	4	16.4	9.2	4.7	17	2.5	1.5

Fungus counts by the plate method ranged from 0 to 473 colonies per plate. The average count for the year (50 exposures) was 36 colonies per plate, for a 12 min. exposure. Table II summarizes these results in terms of weekly averages. As in slide counts, there were marked variations in the number of colonies obtained on different exposures, but no seasonal trends were noted.

The lowest counts were obtained almost invariably from the plates exposed at the seashore, and the highest occurred in the suburban area, the results in the business section being intermediate (Table II).

A total of 1,824 fungus colonies were counted in the plates exposed in the suburban area. Of these 71 per cent were identified morphologically, 17 per cent could not be identified, and 12 per cent were overgrown or could not be subcultured. Forty-three different fungus genera were identified. Among the identified *so-called* genera we have listed three groups which are not true genera (Table III). *Yeast-like* includes Candidas, other white yeast-like forms which did not produce hyphae, and colored yeast-like organisms. The fungi in group 5 A 35 show very similar macroscopic and microscopic characters among themselves. A similar resemblance can be noted among the fungi which constitute group 5A 60. Both produce large dark pycnidia- or perithecia-like bodies which resisted all attempts to make them produce spores, even to cultivation on bits of sterilized leaves, stems, etc. The most numerous genera of fungi in order of appearance were: 5 A 35, Hormodendrum, 5A 60, Basisporium, Penicillium, Phomopsis, yeast-like, Phoma, Aspergillus, Fusarium, Pullularia, Helminthosporium, Stemphylium, and Pestalozzia (see Table III). These genera make up 1,184 colonies, or 65 per cent of the total fungi identified. Other genera which appeared less frequently were Ustilago, *Monilia sitophila*, Stachybotrys, Diplodia, Heterosporium, Zygosporium, Spondylocladium, Trichoderma, Spicaria, Rhizopus, Pringsheimia, Alternaria, Verticillium, Periconia, Fusidium, Cladosporium, Clasterosporium, Cephalosporium, Trichosporum, Sporotrichum, Scopulariopsis, Tetraploa, Pyrenochaeta, Mucor, Mycogone, Mycosphaerella, Graphium, Beltrania, Acrotheca, and Trichosporon.

TABLE III. MOST COMMON GENERA OF FUNGI IN FIFTY WEEKS

GENERA	NO. OF COLONIES	NO. OF APPEARANCES
1. 5A 35	298	26
2. Hormodendrum	219	37
3. 5 A 60	141	12
4. Basisporium*	97	28
5. Penicillium	96	12
6. Phomopsis	72	15
7. Yeast-like†	50	22
8. Phoma	41	11
9. Aspergillus	38	16
10. Fusarium	34	11
11. Pullularia	30	9
12. Helminthosporium‡	24	11
13. Stemphylium	23	11
14. Pestalozzia	21	9
Total	1,184	

These fourteen main genera make up 64.9 per cent of the total number of isolated colonies (1,823), or 91 per cent of the total number of identified colonies.

*Basisporium includes Nigrospora.

†Includes pigmented and nonpigmented yeasts and Candida.

‡Includes the genus known as Curvularia.

There were no particular seasonal relationships in the appearance of individual fungus genera, with the sole exception of fungus 5A 60 which appeared only from December to June. The genus Alternaria was twenty-eighth

in the order of number of appearances and accounted only for 0.2 per cent of the colonies that were identified. Table III shows that the fungus groups dominant in total number of colonies are also dominant in number of appearances. This had been previously noted by Collier and Ferguson[8] in Brunswick, Ga.

The pollen counts were uniformly low throughout the year. Pollen counts of over 6 grains per square centimeter were obtained in only twenty-seven days; these counts occurred on four consecutive days in June and on three days in August, while the other "high" counts occurred on isolated days throughout the year. Because of the low pollen counts, no attempt was made at a botanical classification of the pollen grains. A set of slides exposed in October, 1956, was examined by Mr. Oren C. Durham. Grass pollens were most frequently found, and a few tree pollens were noted.[9]

The slides were studied daily for the presence of recognizable insect particles from Sept. 15, 1956, to Jan. 31, 1957. A total of 439 particles were identified in 133 days, rendering an average daily count of 3.3 particles.

CLINICAL RESULTS

There were 223 cases of bronchial asthma and 77 of allergic rhinitis in the 300 patients studied. The composition of the series by age, sex, and duration of illness is summarized in Table IV. The predominance of children and young adults reflects the selection of cases showing clear-cut extrinsic allergy. The high incidence of reactivity to skin test allergens is shown by the reaction of the group to house dust (Endo's dust): 17 per cent showed positive scratch tests; 16 per cent gave 4+ intradermal, 57 per cent, 3+; 8.67 per cent, 2+; and only 0.67 per cent (two cases gave 1+ or negative reactions to this potent antigen).

TABLE IV. COMPOSITION OF CLINICAL SERIES OF 300 CASES (AVERAGE AGE–20.7 YR.; AVERAGE DURATION OF ILLNESS–7.1 YR.)

	BRONCHIAL ASTHMA—223 CASES AVERAGE AGE—18.2 YR. AVERAGE DURATION OF ILLNESS—7.9 YR.		ALLERGIC RHINITIS—77 CASES AVERAGE AGE—23.2 YR. AVERAGE DURATION OF ILLNESS—6.3 YR.	
	MALES (100 CASES)	FEMALES (123 CASES)	MALES (34 CASES)	FEMALES (43 CASES)
Age range	3-58 yr.	3-65 yr.	5-46 yr.	4-47 yr.
Average age	13.7 yr.	22.8 yr.	22.0 yr.	24.4 yr.
Duration of illness	1-39 yr.	1-40 yr.	—	1-20 yr.
Average duration	6.1 yr.	9.7 yr.	5.7 yr.	7.0 yr.

Seasonal Variations of Allergic Symptoms.—Practically all the patients had year-round allergic symptoms. Sixty per cent observed no seasonal differences at all; 5.7 per cent had year-round symptoms but claimed to be worse in the summer; 26 per cent had symptoms in any season but had exacerbations in the winter (many of the latter were related to respiratory infections). Only one patient in the 300 claimed to have symptoms in one particular month of the year, the reason for which was not explained.

Fungus Allergy in Individual Patients.—A history of exacerbation of allergic symptoms on entering damp places, such as old homes and closets, or handling objects, such as moldy clothes or mildewed foods, was considered suggestive of fungus allergy.[10] All the patients were tested intracutaneously with extracts from cultures of eight genera of fungi, which include about 60 per cent of the genera identified in Puerto Rico, and reactions were graded as strongly positive (3 to 4+), moderately positive (2+), weakly positive (1+), and negative. On this basis, the following criteria for the presence and importance of fungus allergy in individual patients were established:

1. Fungus allergy important:

   Positive history and strongly positive skin tests.

2. Fungus allergy contributory:

   Positive history and moderately positive skin tests.

3. Fungus allergy questionable:

   Positive history and weakly positive skin tests, or negative or doubtful history and strongly positive skin tests.

4. Fungus allergy absent:

   Positive history with negative skin tests, or negative history with moderate or weak positive skin tests, or negative history with negative skin tests.

Fungus allergy was considered an important factor in 6.3 per cent of the entire group (asthma and allergic rhinitis), and a contributory factor in 19.7 per cent; thus, fungus allergy appeared to be significant in 26 per cent or about one fourth of all cases. Twenty per cent had questionable fungus allergy. In 54 per cent no fungus allergy was found but there was clinical and skin test evidence of allergy to universally occurring allergens such as dust, other inhalants, and foods.

Comparison of Allergic Symptoms in the Same Patients Living In and Out of Puerto Rico.—Sixty-five of the 300 patients had traveled to the temperate zones (United States or Europe) after the onset of their allergic symptoms (Table V). Forty-three patients claimed that there was no change in symptoms in or out of Puerto Rico, and showed almost identical evidence of fungus allergy (25.6 per cent) as that of the entire group of 300 patients. Of the 22 patients who improved while away from Puerto Rico, 50 per cent appeared to show important or contributory fungus allergy.

TABLE V. INCIDENCE OF FUNGUS ALLERGY

	SYMPTOMS IN AND OUT OF PUERTO RICO (43 CASES)		SYMPTOMS ONLY IN PUERTO RICO (22 CASES)		ENTIRE SERIES (300 CASES)	
Important	4.6%	25.6%	9.1%	50%	6.3%	25%
Contributory	21.0%		40.9%		19.7%	
Questionable	23.2%		13.6%		20.3%	
None	51.2%		36.4%		53.7%	

DISCUSSION

The survey here reported demonstrates that fungus spores are the most common air-borne allergens of Puerto Rico. The fungus spore count, both by the slide and colony-plate methods, show day-to-day variations but there are no demonstrable seasonal differences. In the absence of any relationship to corresponding meteorological data, the high fungus counts in the suburban region could be related to the rich vegetation in the area involved, which would favor fungus growth. Similar results were obtained by Collier and Ferguson[8] in the Brunswick, Ga., area and by Wallace, Weaver and Scherago[11] in the Lexington, Ky., area.

We found an average of 36.5 colonies per plate throughout the year, while Myers,[12] in Honolulu, reported a total of 1,803 colonies in 248 plates exposed over a period of sixteen months, giving a lower average of 13.6 colonies per plate. Since both these surveys were done according to the standard procedure recommended by the Sub-Committee on Mold Survey of the American Academy of Allergy, the results are comparable.

It is interesting to note that the genus Alternaria appeared in Puerto Rico only five times in fifty exposures, and that it produced less than 1 per cent of the total number of colonies isolated. This is in striking contrast to the situation in Canada,[13] the United States,[14] and Mexico and Cuba,[15] where Alternaria is one of the genera appearing most frequently. However, the situation in other tropical countries, such as Costa Rica,[16] Brazil (Rio de Janeiro),[17] and Honolulu,[12] where Alternaria made up less than 1 per cent of the total fungi isolated, is similar to that of Puerto Rico. We isolated Actinomycetes. Targow and Plunkett[18] found them to make up 6 to 7 per cent of the total count in their study carried out in 1940-1945 in the Los Angeles, Calif., area. They remark that, in other current reports, Actinomyces make up 1 per cent or less of the total fungi isolated. They consider this fact difficult to understand in view of the widespread distribution of these organisms in the soil. It must be noted that they employed Sabouraud's medium and a thirty minute exposure period. Gordon[19] in his 1953-1954 survey of the Atlanta, Ga., area found that Streptomyces ranked with Alternaria and Hormodendrum as the three consistent dominants as to total number of isolates, frequency of appearance, and frequency of dominance on any one day. He used twenty minute exposure periods and Czapek-Dox or potato dextrose agar. However, Myers[12] in Honolulu and Quintero-Fossas in Cuba[15] do not list Actinomycetes at all, and Salazar Mallen in Mexico[15] and Evans and Ruiz[16] in Costa Rica report them as comprising only 2.1 and 1.5 per cent of the total fungi isolated. As suggested by Targow and Plunkett,[18] this discrepancy may be due to the fact that slow-growing Actinomycetes may be masked by more luxuriant growers and thus pass unnoticed.

Hormodendrum, Penicillium, and Aspergillus seem to be ubiquitous. Assuming that pollen counts of 6 to 11 grains per square centimeter are necessary to cause symptoms in allergic patients,[20] the pollen counts obtained in this study appear to be too low to have clinical significance.

Plans capable of producing allergenic pollen do occur on the island. Durham has identified some ragweed plants on the north shore about sixty miles from San Juan. In view of the low pollen counts, residence in the immediate vicinity of any such plants would be necessary for the development of symptoms in sensitive persons. We know of no instance of clinical ragweed sensitivity in patients who never left the island.

The lack of definite seasonal exacerbations in the patients studied is in agreement with the similar absence of differences found in the air-borne allergen survey. This is in sharp contrast with the experiences with seasonal asthma and hay fever in the temperate zones. Fungus allergy appears to be a significant factor in the over-all problem of the patients studied. The doubled incidence of fungus allergy in those patients whose symptoms were improved when they were in the temperate zones suggests the importance of fungi as regional allergens in Puerto Rico. In over half of the series, the problems encountered were similar to those found in allergy practice in any part of the world. Rarely have we seen isolated instances of sensitivity to unusual allergens, such as bat excrement, pollen from the mango tree, or foods, such as the mango and guava. These patients constitute an insignificant portion of the allergic population of Puerto Rico. The findings of insect particles on the slides suggests the possibility that there may be cases of hypersensitivity to insect allergens on the island.

Allergic patients coming to Puerto Rico from the United States should generally expect relief of symptoms due to pollen sensitivity. Patients with fungus allergy may have persistence of their symptoms; for those living on the island, residence in the immediate vicinity of the ocean may be beneficial. The year-round pleasant, warm weather would make the island a satisfactory place for patients with chronic asthma and respiratory infection who develop exacerbations of their disease in cold and inclement weather. The findings of this study could well be applicable to other tropical islands geographically similar to Puerto Rico.

SUMMARY

1. A one-year aeroallergen survey of San Juan, Puerto Rico, is presented.
   a. Fungus spores are the most commonly found aeroallergens in Puerto Rico.
   b. The pollen counts are too low to be clinically significant.
   c. There are marked day-to-day variations in the fungus and pollen count, but there are no seasonal differences.
   d. The most commonly encountered fungi are: Hormodendrum, Basisporium, Penicillium, and two unidentifiable groups.

2. Clinical findings of 300 patients with respiratory allergy living in Puerto Rico are evaluated.
   a. There is no seasonal variation of allergic symptoms.
   b. Fungus allergy appears to be a significant factor in one fourth of all patients and in half of those patients who traveled to the temperate zones and obtained relief of symptoms.

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RELATIONSHIP OF INCREASED HISTIDINE DECARBOXYLASE ACTIVITY TO BORDETELLA PERTUSSIS VACCINE SENSITIZATION OF MICE

Richard W. Schayer, Ph.D., and Oswald H. Ganley, Ph.D., Rahway, N. J.

Bordetella pertussis vaccine is capable of rendering certain strains of mice highly sensitive to histamine and serotonin, to anaphylactic shock, and to a number of other agents and conditions.[1]

In this paper it is shown that injection of mice with pertussis vaccine increases histidine decarboxylase activity in certain tissues. Evidence is presented which suggests that this biochemical change is related to the phenomenon of sensitization.

Histamine is mobilized in mammals by two principal processes: one is by rapid release from the bound condition; the second process is by new synthesis of histamine catalyzed by an adaptive form of histidine decarboxylase.* This enzyme is activated by a number of different stimuli.[2-6]

Our findings lead to the hypothesis that pertussis sensitization is due to initiation and maintenance of new histamine synthesis in or near sensitive cells of mouse lung, and possibly of other tissues, without concomitant increase in adrenal steroid output. The continued exposure of these cells to newly synthesized histamine may produce a reversible alteration of the cell which can be rendered irreversible and lethal by injection or release of histamine or serotonin.

MATERIALS AND METHODS

Determination of Histidine Decarboxylase Activity.—The method in detail has been published.[5] Briefly, it consists of breaking tissue cells by repeated freezing and thawing, then centrifugation at high speed. Aliquots of the supernate are incubated with 10γ C^{14}L-histidine for three hours at 37° C. under nitrogen. C^{14} histamine formed is determined by isotope dilution assay; nonisotopic carrier histamine is added, extracted, converted to dibenzenesulfonylhistamine (BSH), recrystallized, and counted in a liquid scintillation counter. Results are calculated as counts per minute per 100 mg. BSH.

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*It has been shown by two techniques that there is a correspondence between in vitro histidine decarboxylase assays and in vivo histamine synthesis.[4, 5] Histamine formed by inducible histidine decarboxylase is called induced histamine to distinguish it from the bound histamine which may be present in many tissues.

Blank values generally are 30 to 50 units, control lungs 20 to 60 units after subtraction of blank, and lungs of pertussis-treated mice generally three to eight times the values of the controls. In this paper, for comparison of experiments done at different times and for ease in evaluation of percentage increases of enzyme activity, all results were recalculated so that the average of the control samples arbitrarily equals 100.

Determination of Blood Corticosterone Levels.—The method of Silber, Busch, and Oslapas[7] was used.*

B. PERTUSSIS *Sensitization.*—Mice (Tumblebrook strain and CF_1 strain) were sensitized to serotonin or histamine toxicity by the intraperitoneal injection of 2 billion smooth killed *B. pertussis* cells. After a suitable interval as indicated, sensitivity was measured by the administration of 10 mg. per kilogram of serotonin intravenously or 25 mg. per kilogram of histamine intraperitoneally. In anaphylaxis studies, the *B. pertussis*-treated mice were passively immunized to crystalline egg albumin by the intraperitoneal injection of 0.15 to 0.2 ml. of mouse anti-egg albumin ascitic fluid (250γ antibody nitrogen per milliliter), and one hour thereafter were challenged intravenously with 0.5 mg. of commercial crystalline egg albumin in 0.2 ml. saline. When histidine decarboxylase activity was assayed, mice were killed three or four days after pertussis injection and the pooled tissues of several mice, usually three, assayed.

Staphylococcus Vaccine.—Staphylococcus aureus (Smith strain) was grown for twenty-four hours in brain heart broth and then autoclaved for fifteen minutes and diluted 2 parts vaccine with 1 part of saline. Mice were injected intraperitoneally with 0.25 ml.

Typhoid Vaccine.—Combination typhoid-paratyphoid vaccine was purchased from Lederle Laboratories. Mice were injected intraperitoneally with 0.25 ml.

Proteus Vaccine.—Proteus vulgaris strain 1810 was grown for eighteen hours in brain heart broth and, after the addition of formalin (final concentration 1 per cent), incubated for three days at 37° C. Mice were injected intraperitoneally with 0.25 ml.

Freund's Adjuvant.—Freund's adjuvant consisted of 2 parts of White Oil N.F. containing 2 mg. per milliliter *Mycobacterium phlei,* 1 part Arlacel A.†

RESULTS

Effect of B. PERTUSSIS *Vaccine on Histidine Decarboxylase Activity of Mouse Lung.*—The original observation that treatment of mice with pertussis vaccine results in increased lung histidine decarboxylase activity three days after injection has been reported.[2] Results for TF and CF mice are shown in Experiments A and B, Table I.

*The authors are indebted to Dr. R. H. Silber and Mr. Paul Arcese for the corticosterone assays.

†Atlas Powder Co., Wilmington, Delaware.

TABLE I. EFFECT OF VARIOUS SUBSTANCES AND CONDITIONS ON HISTIDINE DECARBOXYLASE ACTIVITY OF MOUSE LUNG

EXPER.	TREATMENT*	HISTIDINE DECARBOXYLASE ACTIVITY (RELATIVE TO CONTROLS = 100)†	AVERAGE
A	Control (TF mice‡)	58, 101, 103, 138	100
	Pertussis	310, 650, 1030, 1100	770
B	Control (CF mice)	73, 78, 117, 132	100
	Pertussis	516, 652, 698, 838	676
C	Control	54, 96, 110, 110, 130	100
	Pertussis	266, 460, 666, 1930	830
	Pertussis (heated)	168, 416, 504, 1290	595
D	Control liver	79, 91, 94, 106, 130	100
	Pertussis liver	161, 167, 179, 192	175
E	Control leg muscle	83, 90, 96, 114, 117	100
	Pertussis leg muscle	100, 129, 163, 206, 219	163
F	Control spleen	43, 96, 161	100
	Pertussis spleen	175, 408, 703	429
G	Control skin	96, 100, 104	100
	Pertussis skin	89, 107, 117	104
H	Control	84, 105, 111	100
	Staphylococcus vaccine	43, 80, 113	79
I	Control	92, 103, 105	100
	Typhoid vaccine	23, 23, 99	48
J	Control	79, 108, 113	100
	Proteus vaccine	117, 160, 188	155
K	Control	88, 93, 119	100
	Freund's adjuvant (oil only)	133, 135, 135	134
	Freund's adjuvant + *M. phlei*	560, 737, 781	693
L	Control	88, 100, 112	100
	M. phlei in aqueous medium	53, 76, 132	87
	Freund's adjuvant (oil only)	68, 115, 132	105
	Freund's adjuvant + *M. phlei*	438, 485, 805	576

*See text for full details.

†For explanation, see Methods.

‡TF mice used, and lungs taken for assay three or four days after injection unless otherwise noted.

Histidine Decarboxylase Activity of Mouse Lung at Various Intervals After Injection of B. PERTUSSIS *Vaccine.*—Mice were killed on various days following inoculation; results are shown in Fig. 1. In the first curve, one of the early experiments, maximum enzyme activity was observed on the third day. The second curve is from data obtained several months later; the maximum was obtained on the fourth day. In accordance with these findings, in the earlier experiments, animals were tested three days after pertussis injection, and, in later experiments, four days after injection.

TF mice injected with pertussis vaccine reach maximum sensitivity to passive anaphylaxis on the third or fourth day; sensitivity is reported to persist for eight to ten days.[8] Sensitivity to injected serotonin and histamine also becomes maximal on about the third or fourth day but persists somewhat longer; sensitivity to serotonin appears to be lost between the twelfth and eighteenth day[9] while sensitivity to histamine is reported lost between the fourteenth and twenty-eighth days.[8] Fig. 1 shows that histidine decarboxylase activity of lung reaches a maximum on the third or fourth day, is slightly elevated on the eighth day, and at the thirteenth day is no longer detectable; no detailed exploration of the time of return of normal enzyme activity was made. Thus, there is a fundamentally good agreement between the enzyme activity curve and the periods of

sensitization. Discrepancies may be explicable by one or more of the following: (a) the in vitro enzyme activities may not correspond exactly to in vivo histamine synthesis, (b) after sensitivity of cells is established, a very low rate of histamine synthesis may be adequate for maintenance, (c) biological sensitivity may be influenced by all tissues where enzyme activation occurs; the enzyme time curve was determined for lung only, (d) histamine and serotonin may act primarily on different tissues, (e) the exact time when sensitization is lost may be subject to more precise evaluation, (f) enzyme activities and sensitivity studies were done under different conditions.

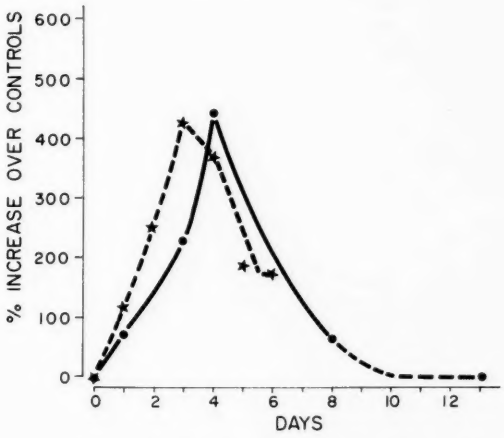

Fig. 1.—Curves of two experiments showing histidine decarboxylase activity of mouse lung at various intervals after injection of *B. pertussis* vaccine.

Effect of Pertussis Vaccine on Corticosterone Levels of Mouse Blood.—Table II shows that treatment of mice with pertussis vaccine results in a rapid increase in the blood concentration of the principal adrenal steroid of this species, corticosterone; the levels then return to normal at some time between the seventh and sixteenth hours. This early rise is probably an endotoxin effect.* Comparison of the data of Table II with those of Fig. 1 shows that during the greater part of the period of increased histidine decarboxylase activity, there is no compensation due to increased adrenal steroid output.

Effect of Heat.—Heating pertussis vaccine at 56° C. for thirty minutes inactivates a toxic ingredient but does not destroy the sensitizing factor.[10] The data of Experiment C (Table I) show that heated vaccine is capable of increasing histidine decarboxylase activity of mouse lung.

Effect of Pertussis Vaccine on Histidine Decarboxylase Activity of Other Mouse Tissues.—Under conditions in which a sixfold increase in lung histidine

*Pertussis vaccine contains endotoxin; like other endotoxins it causes a rapid increase in histidine decarboxylase activity which reaches a maximum in about six hours and returns essentially to normal in a day or less.[4,6] The vaccines of typhoid, proteus, and staphylococcus show this rapid effect also. It is a typical systemic stress reaction; the time of increased histidine decarboxylase activity corresponds closely to the time of increased corticosterone output. Hence neither the histamine nor the adrenal steroid is markedly excessive during this period. It is the protracted increase of histamine production, which becomes uncompensated at some time between the seventh and sixteenth hour, which is believed to cause sensitization.

TABLE II. CORTICOSTERONE LEVELS OF MOUSE SERUM AT VARIOUS INTERVALS AFTER INJECTION OF *B. pertussis* VACCINE

TIME (HR.)	CORTICOSTERONE (γ/ML.)	
	EXPER. I	EXPER. II
0 (Control)	0.32	0.34
0.5	0.44	0.42
2	0.66	0.58
4	0.50	0.58
7	0.40	0.44
16	0.24	0.20
24	0.14	0.30
48	0.21	0.30
72	0.12	0.12
96	0.22	0.26

decarboxylase was obtained, liver and leg muscle from the same mice showed only a slight increase in enzyme activity. Values for spleen were elevated although quite variable; skin was unaffected (Experiments D, E, F, and G).

Absence of Prolonged Activation of Histidine Decarboxylase by Various Other Bacterial Products.—Endotoxins produce a rapid increase in histidine decarboxylase activity which reaches a maximum in about six hours. Thereafter it returns essentially to normal in one day[4] and is not elevated on the third or fourth day after injection. *Mycobacterium phlei* in aqueous medium, and typhoid, proteus, and staphylococcus vaccines had no significant effect on enzyme activity of mouse lung three or four days after injection (Table I, Experiments H, I, J, and L). Thus, the prolonged activating effect of pertussis vaccine on the enzyme is not shared by some other bacterial products.

Studies on Freund's Adjuvant.—If another substance were to be found which induced a prolonged increase in mouse lung histidine decarboxylase and also produced sensitization, the hypothesis would be materially strengthened. Freund's adjuvant containing killed *Mycobacterium phlei* was tested because, like pertussis vaccine, it has biological effects which seem to be independent of the adjuvant action. *M. phlei*, Freund's adjuvant without *M. phlei*, and complete Freund's adjuvant were injected intraperitoneally in 0.25 ml. quantities. Results of histidine decarboxylase assays are shown in Experiments K and L, Table I. Only the Freund's adjuvant containing killed *M. phlei* produced an increase in enzyme activity.

TABLE III. CORTICOSTERONE LEVELS OF MOUSE SERUM AT VARIOUS INTERVALS AFTER INJECTION OF COMPLETE FREUND'S ADJUVANT

TIME (HR.)	CORTICOSTERONE (γ/ML.)	
	EXPER. I	EXPER. II
0 (Control)	0.14	0.20
0.5	0.66	0.52
2	0.56	0.58
4	0.72	0.60
7	0.38	0.36
16	0.36	0.50
24	0.52	0.30
48	0.46	0.42
72	0.26	0.32

Since complete Freund's adjuvant produced a sustained increase of histidine decarboxylase activity in mouse lung, the effect of the adjuvant on sensitization of mice was tested. Mice were injected intraperitoneally with 0.25 ml. of the adjuvant and were challenged three days later with 75 mg. per kilogram serotonin base intravenously. There was no evidence of sensitization; all mice survived. In this case, mice showed increased histidine decarboxylase activity of the lung but were not sensitized.

To maintain the hypothesis that sensitivity is due to the induced enzyme, it was necessary to obtain evidence for adrenal stimulation other than the obviously stressed appearance of Freund's adjuvant-treated mice. Table IV shows that blood corticosterone levels of Freund's-treated mice are rapidly increased and that the augmented production is still present three days after injection. Thus, Freund's adjuvant probably does not sensitize because it stimulates a large output of adrenal steroids.

TABLE IV. EFFECT OF HYDROCORTISONE ON SENSITIZATION WITH *B. pertussis* VACCINE

COMPOUND	TIME BETWEEN CESSATION OF HYDROCORTISONE TREATMENT AND CHALLENGE (HR.)	SURVIVAL/TOTAL	PER CENT SURVIVAL
Control	24	8/40	20
Hydrocortisone		27/35	77
Control	48	6/30	20
Hydrocortisone		21/29	72
Control	72	7/29	24
Hydrocortisone		11/29	37
Control	96	10/29	34
Hydrocortisone		9/26	34

Effect of Hydrocortisone on Sensitization.—If prolonged exposure of inadequately protected cells to newly synthesized histamine is the cause of sensitization, then protection of these cells from the effects of histamine should prevent sensitization. Mice were given a diet containing 60 mg. per kilogram of hydrocortisone starting one day before *B. pertussis* injection and for three days thereafter. Hydrocortisone is completely eliminated from the body of the mouse within a few hours after ingestion.* The mice were then challenged with serotonin at various intervals after cessation of hydrocortisone intake. The data of Table IV show that mice are protected from death for forty-eight hours after cessation of treatment with hydrocortisone. The sensitivity then returns, becoming essentially complete in three days. These results are in excellent agreement with those predicted on the basis of the hypothesis.†

*Injected corticosterone, and corticosterone synthesized endogenously after injection of the precursor, are virtually undetectable in mice within two hours.[29] Hydorcortisone behaves similarly (Darrach, M., personal communication). The gastrointestinal tract of the mouse empties in two to six hours after feeding (Bosshardt, D., unpublished data). Thus about eight hours is the maximum time ingested hydrocortisone might be expected to remain in the body. This conclusion has been confirmed by feeding C^{14} hydrocortisone to mice (Ganley, O. H., unpublished data).

†In rats, adrenal steroids suppress histidine decarboxylase activity of lung[30]; this effect has not been detected in mice (Schayer, R. W., unpublished data) although several steroids have been tested. It is, therefore, unlikely that inhibition of pertussis sensitization by hydrocortisone is related to suppression of histidine decarboxylase activity.

DISCUSSION

It is our belief that the sensitizing properties of pertussis vaccine in the mouse are due to its ability to initiate a prolonged increase in the rate of histamine synthesis in lung, and possibly other tissues, without a concomitant increase in adrenal steroid output. Because of the long time required for each assay, lung is the only tissue studied extensively. Other tissues may also participate in sensitization* and may differ from lung in time of maximal enzyme activity and in length of activation. The results on lung are considered as an example of the basic mechanism leading to sensitization and suggest that lung may be an important shock tissue, but not necessarily the only one.

The results of all of our experiments and the results of many experiments reported by other laboratories are compatible with the hypothesis stated above.

Kind[1] has listed the compounds to which pertussis-treated mice do not show increased sensitivity. They are epinephrine, methacholine, carbaminocholine, and Compound 48/80. According to the hypothesis, epinephrine should not be harmful, because it antagonizes histamine. The choline esters are known to release epinephrine and norepinephrine from the adrenals.[11] Compound 48/80 disrupts mast cells and releases histamine in the rat and several other species. In the mouse, however, it is not a very effective mast cell disruptor[12] and there is evidence that the toxic effect of Compound 48/80 in mice is due to factors other than histamine release.[13]

Of the substances listed by Kind[1] other than histamine and serotonin to which pertussis-inoculated mice are susceptible, peptone may act through release of amines; all the other substances listed by Kind contain bacterial endotoxins or may have endotoxin-like properties. In view of the evidence for participation of induced histamine in endotoxin shock[4] it would be expected that a histamine-sensitive animal would suffer a delayed death from a low dose of endotoxin; not a rapid death as in anaphylaxis. These time relationships are observed in pertussis-inoculated mice.

The hypothesis is compatible with numerous reports of differences among various strains of mice in sensitivity to histamine, serotonin, and anaphylaxis. These differences may be explicable in terms of the rate and duration of histamine synthesis in various tissues, whether such tissues are specific targets for injected histamine or serotonin or for substances released in anaphylaxis, whether adrenal steroids effectively protect the target organ, the number and location of mast cells, their content of histamine and serotonin, the susceptibility of mast cells in various locations to disruption, the ability of injected or released amines to reach the sensitized cells, the capacity of various tissues for inactivating histamine and serotonin, availability of protective compounds, such as norepinephrine, from the sympathetic nerve endings, and to numerous other factors.

*The degree of sensitization of cells is, of course, not necessarily proportional to the per cent increase of histidine decarboxylase activity in a tissue over normal values. Hence any tissue, in which a significant, prolonged increase of enzyme activity is observed, must be considered as a possible contributor to the sensitization of the mouse.

Only one other change in enzyme activity seems to have been reported in pertussis-sensitized mice. Kind and co-workers[14] found a reduction of histaminase activity of mouse lung but were unable to confirm this at a later date.[1] Niwa and co-authors[15] published similar findings in tissues of pertussis-treated mice and rats. It is doubtful that a drop in histaminase activity could be of significance in sensitization. Histaminase is of minor importance in histamine destruction in mice; methylation is the principal means of inactivation in this species.[16] Furthermore, mice are sensitized to serotonin which is not a substrate of histaminase.

The failure of Fishel[17] to find increased histamine concentrations in tissues of sensitized mice does not oppose the hypothesis. The concentration of "bound" histamine, which is protected from destruction, may be quite high in some tissues. Induced histamine is not bound and is rapidly destroyed. Hence an increased rate of synthesis of the latter might not produce a significant rise in tissue histamine. The efficacy of the newly synthesized histamine may lie not in quantity but in site of formation and length of exposure of cells to it.[4]

Pertussis vaccine seems to contain one or more substances which is retained in lung, probably in reticuloendothelial cells[18]; like other activators of the reticuloendothelial system tested, it also activates histidine decarboxylase.[4, 5] The period of retention of the stimulant in these cells may be reflected by the duration of increased enzyme activity.

In pertussis-vaccinated mice, certain histamine-sensitive cells, such as those of smooth muscle and endothelium, may undergo changes due to a prolonged state of abnormal permeability of the membrane. Eyring and Dougherty[19] have discussed the nature and significance of the permeable state, the dangers to the cell of remaining in the permeable state for prolonged periods, and reasons for implicating histamine in permeability changes.

As a result of its initiation and maintenance of uncompensated histamine synthesis, pertussis vaccine presumably increases the need of certain tissues for adrenal steroids to restore the normal permeability. This state could account for the observations that pertussis-vaccinated mice, despite evidence of normal adrenal function,[20–22] behave in many respects like adrenalectomized mice.[23] Adrenalectomy renders mice histamine-sensitive by removal of histamine-antagonizing hormones[24]; pertussis vaccine may render mice histamine-sensitive by inducing endogenous histamine synthesis and thus reducing the threshold concentration of exogenous histamine required to cause irreversible changes.

Thus, the hypothesis on the mechanism of pertussis sensitization is compatible with the results of a large number of highly diversified experiments. Some of these, the time curve (Fig. 1) and the hydrocortisone protection experiments, have submitted the present interpretation to rigorous tests. The authors are not aware of a single well-established observation which opposes the conclusions of this paper.

Finally, the hypothesis is logical. If histamine and serotonin, the latter having histamine-like properties in rats and mice,[25] are unique in ability to produce damage in a pertussis-sensitized mouse by direct action, then one of

these amines, if mobilized in a slow, prolonged manner, in sublethal quantities, should also be capable of effecting the initial sensitization. Induced histamine fully satisfies these requirements; attempts to demonstrate increased serotonin production in cases in which increased histamine synthesis occurs have been unsuccessful.[26-28]

SUMMARY

Injection of mice with *Bordetella pertussis* vaccine, a procedure which renders certain strains of mice highly sensitive to histamine, serotonin, and anaphylactic shock, also leads to increased histidine decarboxylase activity in lung during a period which corresponds closely to the time of sensitivity.

The hypothesis is presented that pertussis sensitization is caused by prolonged exposure of certain cells of lung, and possibly of other tissues, to newly-synthesized histamine. In the absence of a significantly increased output of protective hormones from the adrenals, this histamine may produce a reversible alteration of permeability in the membranes of the affected cells and changes in the intracellular organization; then injection or release of histamine and serotonin may result in irreversible damage and death.

A number of experiments are described which are compatible with this hypothesis.

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EXPERIMENTAL EOSINOPHILIA

III. Regional Lymph Node Responses to Reactions of Tissue Sensitization

Sheldon G. Cohen, M.D. Milton Kantor, M.D.,** and Louise Gatto,****
Wilkes-Barre, Pa.

THE ASSOCIATION of the eosinophilic leukocyte with hypersensitivity reactions occurring under varying sets of immunologic conditions has resulted in multiple and divergent views concerning origin, evolution, and function of this cell. Summarization of this subject can be found in several comprehensive reviews and reports.[1-5] The documentation of experimental evidence suggests that hematogenous and tissue eosinophilic granular leukocytes may indeed possess more than a singular role.

An experimental procedure for effecting localized eosinophilia provided the opportunity to evaluate some theories concerned with eosinotactic mechanisms in the immune response. These included dependence upon and/or relationships to: (1) the presence of foreign protein antigen[6] or antibody per se; (2) antigen-antibody union[7-9] and especially resultant antigen-antibody precipitate, soluble antigen-antibody complexes, or a product released[9] or enzyme activated[10] by this reaction; (3) removal of endogenously released histamine,[3-11] and (4) phagocytosis of mast cell granules,[12] directing attention to the serotonin,[13] and heparin,[14] as well as histamine[15] content of this granule.

The occurrence of eosinophilic granular cell responses within regional lymph nodes draining passively sensitized cutaneous sites formed the basis of this investigation. Factors leading to the development of this experimental technique were: (1) the histopathologic finding of eosinophilic cellular infiltrations within peribronchial lymph nodes of rabbits (Fig. 1) that developed pulmonary vasculitis after subanaphylactic passive sensitization,[16-17] (2) reports documenting the accumulation of eosinophils in lymph nodes draining areas of infection or injected foreign material,[5] (3) the rapidity of lymphatic drainage of foreign material from the rabbit foot pad evidenced by the appearance of India ink

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*Associate Professor of Biological Research.

**Chief of Medical Service.

***Student laboratory assistant.

throughout lymph sinuses of popliteal nodes within thirty minutes and by the uptake of antigen by cellular elements of these nodes ten minutes[18] after respective injections, and (4) the early appearance of eosinophils within a few hours after antigenic challenge in actively sensitized animals.

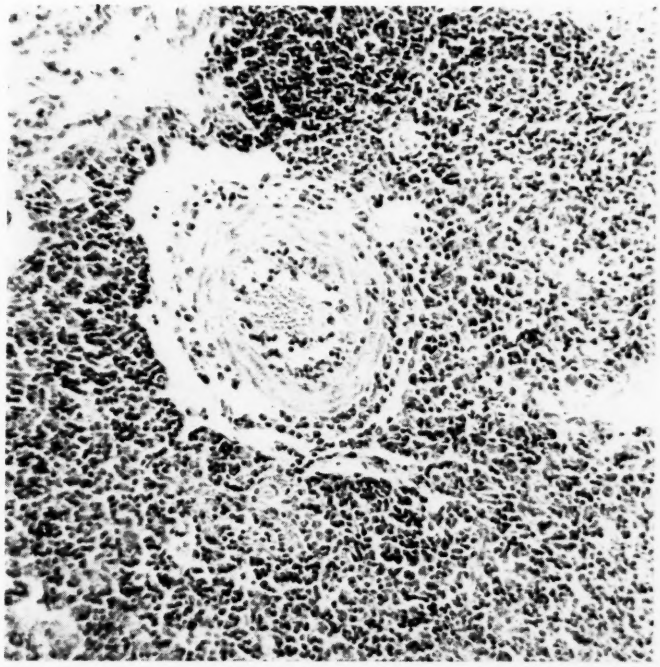


Fig. 1.—Lung section from rabbit subjected to reversed passive sensitization with intravenously administered BSA and corresponding antiserum. A pulmonary arteriole and adjacent peribronchial lymph node are shown. There is very mild involvement of the vessel with endothelial disorganization and a few eosinophilic granular cells infiltrating the intima, media, and periarterial space; however eosinophilic cellular infiltration is seen throughout the adjacent tissue of the lymph node.

MATERIALS AND METHODS

One hundred and ten male albino rabbits, weighing between 2 to 2.5 kilograms, served as experimental animals. These were divided into groups of six with the members of each subjected to one of eighteen experimental procedures described below. All antigen and antiserum injections were given in a total volume of 0.5 ml. directly into the foot pad of one hind extremity of each animal. Antigen consisted of 8 mg. of crystallized bovine serum albumin (Armour) (BSA) dissolved in physiological saline. Corresponding antiserum prepared from blood taken from rabbits immunized with BSA emulsified in both Freund's complete and incomplete adjuvant was given in total doses of 0.2 mg. antibody nitrogen. Four hours after the last foot pad injection the rabbit was sacrificed by air embolism, the popliteal lymph nodes of both hind extremities were immediately exposed, removed, and prepared for histologic study by routine methods of fixation and staining with hematoxylin and eosin. Popliteal nodes draining the untreated contralateral foot pad of each animal served as histologic controls for the study of those taken from the treated extremity.

Experimental Procedures.—

1. Antigen (BSA) administration.

2. Antibody (antiserum to BSA) administration.

3. Antigen antibody administration—BSA and thirty minutes later antiserum to BSA were given.

4. Simultaneous antigen : antibody administration—BSA and corresponding antiserum were given into the foot pad through separate hypodermic needles at the same time.

5. Products of the vitro antigen : antibody reaction—Precipitin test procedures were carried out with antigen and antibody in a total volume of 1 ml. at equivalence. Reactants in saline were placed in a water bath at 37° C. for thirty minutes and in the refrigerator overnight. The absence of excess BSA and antibody in the supernatant was ascertained by negative interfacial tests with corresponding antiserum and antigen, respectively. Experimental groups then received either:

   a. Preformed precipitate—Centrifuged, washed, and resuspended three times in 0.15M NaCl.
   b. Antigen and antibody-free supernatant.
   c. Soluble antigen : antibody complex—Prepared by dissolving precipitate through the addition of BSA in the amount of twenty times equivalence.

6. Unrelated antigen : antibody systems—Each of two rabbits received one of the following:

   a. Antiserum to BSA and one unrelated soluble antigen:
      (1) *Hemophilus pertussis* soluble bacterial antigen (Pertussis UBA, Lilly), 0.5 ml. (0.05 mg. N).
      (2) *Staphylococcus aureus* pepsin digested toxoid (Staphylococcus toxoid, Lederle), 5,000 dermonecrotic units.
      (3) Ragweed pollen *(Ambrosia elatior)* aqueous extract, 0.09 mg. N.
      (4) Tetanus fluid toxoid (Lilly), 0.5 ml.
   b. BSA and one unrelated rabbit antiserum:
      (1) Antiserum to C-reactive protein (CRPA, Schieffelin), 0.5 ml.
      (2) Antiserum to *H. pertussis* (Wyeth), 5,000 units.
      (3) Antiserum to ragweed pollen extract, 0.5 ml.
      (4) Antiserum to *Staphylococcus aureus* toxoid, 0.5 ml.

7. Simultaneous chemical agents—One of the following was given intravenously (iv) immediately prior to BSA, or to antiserum, and at one hour intervals thereafter; and/or directly into the foot pad (ifp) immediately prior to the antiserum.

   a. Antihistaminic agents:
      (1) Tripelennamine (Pyribenzamine, Ciba), 1 mg./Kg. iv and 1 mg. ifp.
      (2) Chlorprophenpyridamine (Chlor-Trimeton, Schering) 5 mg. ifp.

b. Inhibitors of histamine liberation:
   (1) Disodium ethylenediamine tetraacetic acid (EDTA) (Endrate Disodium, Abbott), 15 mg. ifp.
   (2) Iodoacetic acid (Nutritional Biochemicals), 0.5 mg. ifp.
c. Antiserotonin agent—methyl lysergic acid butanol amide tartrate (UML-491, Sandoz), 1.5 mg./Kg. iv and 1 mg. ifp.
d. Antihistamine and antiserotonin agent—Promethazine (Phenergan, Wyeth), 1 mg./Kg. iv and 1 mg. ifp.
e. Antiheparin agent—Protamine (Lilly), 3 mg./Kg. iv and ifp.

8. Histamine, 1 mg. in aqueous solution ifp.
9. Heparin (Liquaemin, Organon), 5 mg. ifp.

RESULTS

Eosinophilic granular cell infiltrations within popliteal lymph node sections were found to occur in three patterns.

1. Marked—Extensive and diffuse uniform infiltrations consisting only of eosinophils completely filling the cortical and medullary sinuses (Fig. 2).

2. Moderate—Uniform collections of eosinophils occupying portions of one or more cortical or lymphoid sinuses.

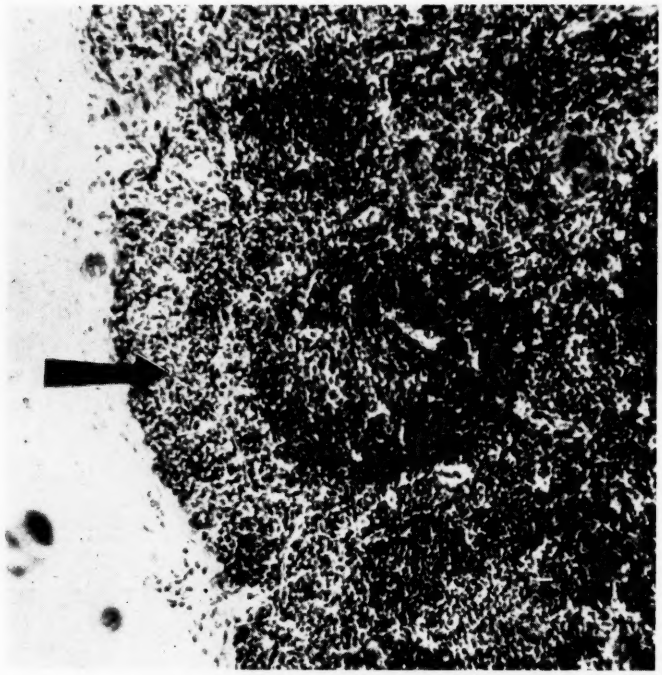
TABLE I

ANTIGEN	ANTISERUM	CHEMICAL AGENT	NUMBER OF RABBITS* EXHIBITING EOSINOPHILIA		
			MARKED†	MODERATE†	NONE OR NORMAL
BSA	None		0	0	6
None	to : BSA		0	0	6
BSA	to : BSA		5	1	0
BSA	to : BSA				
(1) Simultaneous administration			2	2	2
(2) Preformed precipitate			0	1	5
(3) Soluble complexes			0	0	6
(4) In vitro supernatant			0	0	6
H. pertussis UBA	to : BSA‡		0	0	2
Staph. aureus toxoid	to : BSA‡		0	0	2
Ragweed extract	to : BSA‡		0	0	2
Tetanus toxoid	to : BSA‡		0	0	2
BSA	to : CRP‡		0	0	2
BSA	to : *Staph aureus*‡		0	0	2
BSA	to : *H. pertussis*‡		0	0	2
BSA	to : Ragweed‡		0	0	2
BSA	to : BSA	Tripelennamine	6	0	0
BSA	to : BSA	Chorprophen-pyridamine	3	3	0
BSA	to : BSA	Promethazine	3	3	0
BSA	to : BSA	UML-491	4	2	0
BSA	to : BSA	Protamine	3	3	0
BSA	to : BSA	EDTA	4	2	0
BSA	to : BSA	Iodoacetic acid	5	1	6
		Histamine	0	0	0
		Heparin	0	0	6

*Groups of six rabbits except when otherwise indicated by ‡.
†Contralateral popliteal lymph nodes exhibited either no eosinophilia or normal variation.
‡Groups of two rabbits.

3. Normal variations—Sparse eosinophils interspaced and scattered among cellular elements of lymphoid tissue comprising the periphery of cortical nodules or medullary cords, or among the varied cell types found within lymph sinuses.

The results of histologic examinations demonstrating the extent of eosinophilic cellular infiltration of popliteal nodes within members of each experimental group are summarized in Table I.

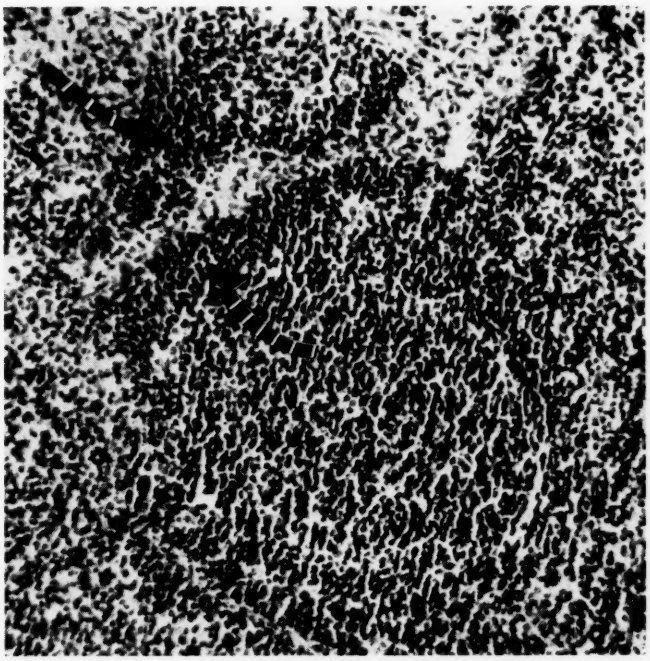


A.

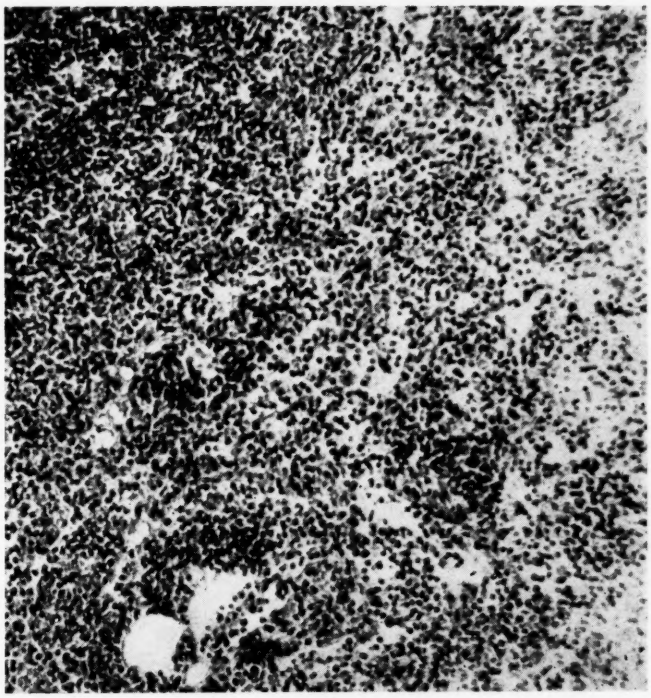
Fig. 2.—Popliteal lymph node taken from a rabbit which had received BSA and, thirty minutes later, antiserum to BSA into the ipsilateral foot pad. Eosinophilic granular cells are seen infiltrating through lymph sinuses. A heavy cellular infiltration is seen completely filling a peripheral sinus in *A*.

COMMENT

In the study of rabbit lymph node cytology, we have attempted to avoid two possible pitfalls in evaluating eosinophilic granular cell components. The first is concerned with normal variations where a few hematogenous eosinophil leukocytes can be found in most lymph nodes.[19] This is especially the case in such animal species as the rabbit where the exposed foot pad is constantly vulnerable to traumatic penetration of foreign and antigenic material. The study of regional nodes draining the untreated contralateral foot pad in each animal verified unequivocal degrees of heavy eosinophilic cellular infiltrations that resulted from in vivo antigen-antibody reactions. The second point of possible error is concerned with the existence of the rabbit "pseudo-eosinophil" cell, a hematogenous leukocyte in which the cytoplasm may appear pink with eosin-containing stains. In addition to control contralateral lymph node study, misinterpretation was avoided by applying standards of identification for the



B.



C.

Fig. 2.—Cont'd. *B*, Eosinophils are seen within a cortical sinus as well. *C*, Eosinophils are seen throughout a medullary sinus.

eosinophilic granulocyte. These included the characteristic bilobed, plump, round, eccentrically-placed, blue-staining nucleus, and coarse, red, cytoplasmic granules.

Although the eosinotactic stimulus of the antigen-antibody reaction is clearly demonstrated, the data do not permit any conclusion concerning the site of such action. Possibilities include (1) an original point of eosinophil collection within the tissue site of sensitization and a subsequent filtering and trapping action by regional nodes on these cells migrating via lymphatic drainage channels, or (2) a direct influx of eosinophils into the cortical and medullary lymph sinuses in response to the union of localized antigen and antibody subsequent to the lymphatic drainage of each from the foot pad. An evaluation of the experimental conditions that led to the development of maximal lymph node eosinophilia favors the latter explanation. The thirty-minute interval between administration of antigen and antiserum allowed a greater opportunity for larger quantities of both free antigen and free antibody to reach the lymphatic channels. It is therefore of significance to note that when antigen and antiserum were introduced simultaneously, permitting partial inter-reaction within the foot pad, the degree of regional node eosinophilic cell accumulations was considerably lessened. Further, when the interval between antigen-antibody administration was lengthened to periods of four to twenty-four hours, the degree of lymph node eosinophilia was considerably lessened and often was an equivocal and inconstant finding. Thus, the union of the small amounts of antibody transported into depots of retained antigen in excess appear to constitute experimental conditions favorable for the development of eosinotactic immune reactions.

The pattern of appearance of eosinophils first within cortical sinuses and later into medullary channels of the node suggests a lymphogenous route of travel. However, the source and site of origin of cellular response to the stimulus of antigen-antibody union is not apparent. This might occur through a liberated eosinotactic factor[9] resulting in cell mobilization from hematogenous sources. Eosinophil development in situ through the conversion of parent lymphocytic cell types[6] through their removal of toxic by-products[20] of the sensitization reaction could also explain the mechanism. Attempts to reproduce this histologic picture through the substitution of free histamine and heparin were unsuccessful. Agents exerting inhibiting effects on histamine liberation and those with antihistaminic, heparin-neutralization, and antiserotonin properties failed to influence eosinophil emergence. This further suggests that neither histamine,[2, 5, 7] heparin,[5, 7, 21] nor serotonin[22] might be the responsible chemotactic influence for the eosinophilia resulting from antigen-antibody union.

Inability to induce local eosinophilia by passive sensitization procedures in the mouse has been reported.[23] This is in contrast to the significant degrees of tissue eosinophilic cell infiltration demonstrated in these experiments. An exact explanation is not apparent but consideration must be given to obvious differences in animal species, experimental techniques, routes of administration of sensitizing and challenging agents and times of specimen collections. However,

current studies in our laboratory would also suggest that the eosinotactic efficacy of immune reactions may be dependent upon the nature of the antigen-antibody system involved.

It has been demonstrated that it is not the sole presence of antigen or of antibody but rather the union of antigen and corresponding antibody that is responsible for the four-hour eosinotactic effect. Although the mode of action is not apparent, these findings do suggest that tissue reactions to the formation of antigen-antibody precipitate or soluble complexes are not directly concerned. The failure of an in vitro serum-liberated product of antigen-antibody mixture to reproduce this histologic alteration indicates the requirement for a tissue site of antigen-antibody union for the initiation of responsible physiologic mechanisms whether they be enzymatic or chemotactic.

SUMMARY

1. The administration of bovine serum albumin (BSA) and corresponding antiserum into the rabbit foot pad resulted in four-hour eosinophilic granular cell infiltrations into the sinuses of regional popliteal lymph nodes.

2. Similar histologic alterations could not be reproduced by BSA or by antiserum to BSA alone, by noncorresponding antigen-antibody systems, histamine, or heparin.

3. The development of lymph node eosinophilia through the action of specific immunologic reactants was not affected by agents with antihistaminic, antiheparin, or antiserotonin properties.

4. The responsible eosinotactic mechanism of in vivo antigen-antibody union has not been determined but there is no evidence to indicate a role for antigen-antibody precipitate, soluble antigen-antibody complexes, or a product of antigen-antibody mixtures liberated in vitro.

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SUPRAVITAL STAINING OF LEUKOCYTES FROM RAGWEED-SENSITIVE INDIVIDUALS

Marino Audia, M.D., and Joseph Noah, M.D., St. Louis, Mo.

THIS STUDY was undertaken to determine whether differences exist in the staining properties of leukocytes from ragweed-sensitive individuals when these leukocytes have been exposed in vitro to ragweed antigen when compared with the results obtained when the same cells have not been exposed to antigen.

Many reports indicate that the white blood cells from sensitive individuals or sensitized animals behave differently in various respects when exposed to a specific antigen, when contrasted with behavior of cells from the same individual or animal not exposed to the antigen. It has been reported, for example, that leukocytes from ragweed-sensitive individuals, when exposed to a final concentration of 1:1000 ragweed extract, undergo a significant degree of lysis,[1] although in another study[2] this could not be verified. A similar study, in which a much higher concentration of antigen was used,[3] reported a marked degree of immobilization and lysis of leukocytes, although in other observers' hands[4] the same technique did not produce significant results.

There has been more agreement on results in bacterial sensitivity studies in which leukocytes from tuberculin-sensitive individuals were exposed to either PPD or to old tuberculin. A significant amount of cytolysis occurs when such leukocytes are exposed to tuberculin.[5] Likewise, exposure to specific antigen of rabbit leukocytes from animals sensitized to ovalbumin or bovine gamma globulin has produced a significant degree of cell lysis when compared with cells processed similarly but not exposed to the antigen.[6]

Since the nuclear staining of leukocytes by neutral red stain is said to occur in dying or dead cells,[7] it was thought that the uptake of neutral red by leukocytes from ragweed-sensitive individuals might serve as a precise indicator of the reaction of such cells to the presence of ragweed antigen. The same principle has been used to determine the death of cells in tissue culture.[8] Accordingly, the following studies were undertaken.

From the Division of Allergy, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri.

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Requests for reprints should be addressed to the senior author whose address is: Apartado 6753, Panama, Republic de Panama.

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METHOD

Blood samples in the amount of 12 to 20 ml. were obtained from the antecubital veins of nonallergic and ragweed-sensitive individuals. Siliconed glassware was used throughout. Enough sodium heparin to wet the inner surfaces of a 20 ml. syringe was used as an anticoagulant.

The nonallergic individuals selected had no history of allergic symptoms and negative skin tests by the conventional intradermal technique with 1,000 PNU per milliliter of ragweed extract. The ragweed-sensitive individuals had hay fever and/or asthma during the ragweed season, with marked skin test reactions to ragweed extract in concentrations of 1,000 PNU per milliliter or less.

Two milliliters (or slightly smaller) aliquots of blood were delivered into tubes of 0.5 cm. in diameter and 10 cm. in length. According to the manner in which the blood samples were treated, they may be grouped as follows:

Group I (16 ragweed-sensitive patients and 10 normal controls)
- Tube 1 No antigen added (control)
- Tube 2 0.10 ml. Coca's solution added (control)
- Tube 3 0.10 ml. of 1,000 PNU ragweed antigen added
- Tube 4 0.10 ml. of 10,000 PNU ragweed antigen added

Group II (10 ragweed-sensitive patients)
- Tube 1 No antigen added (control)
- Tube 2 10 mg. ethylenediamine tetraacetate sodium (EDTA) added
- Tube 3 0.10 ml. of 1,000 PNU ragweed antigen + 10 mg. EDTA added

Group III (4 ragweed-sensitive patients)
- Tube 1 No antigen added (control)
- Tube 2 10 μg/L. histamine added
- Tube 3 0.10 ml. of 1,000 PNU/ml. ragweed antigen + 10 μg/L. histamine added
- Tube 4 60 μg/L. histamine added
- Tube 5 0.10 ml. of 1,000 PNU/ml. ragweed antigen + 60 μg/L. histamine added

All ragweed extracts were in Coca's solution, without glycerine.

These procedures were done at a room temperature ranging from 21° to 24° C. In addition, duplicate sets, except for the first ten samples, were incubated in a water bath at 37° C. for fifteen minutes. All samples were centrifuged at 800 r.p.m. for ten minutes. Most of the supernatant plasma was removed by pipette, leaving a loosely-packed buffy coat of white blood cells and platelets.

Ten microliters of 0.2 per cent neutral red in methanol were placed on clean coverslips and allowed to dry. At intervals of thirty minutes, new preparations were made by placing a loopful of the buffy coat on a coverslip coated with the dried stain and rimmed with petrolatum and immediately inverting the coverslip on an acid-cleaned slide to avoid evaporation and damage to the cells. A preparation showing a substantial number of motile leukocytes was considered satisfactory. Three minutes after exposure to the dye, the cells were examined by phase microscopy. One hundred white cells were

counted and the number obviously stained was recorded. Care was exercised to observe the center of the preparation instead of the periphery in order to avoid areas with a greater concentration of stain.

RESULTS

Rapid staining of nuclei did not occur. No changes were noted in the nuclei of most cells, while the staining of the cytoplasm, ranging in color from light gold to orange, could be noted in an increasing number of cells as observations continued. The few cells in which nuclear staining occurred appeared to be dead. Many of the cells remained quite active after cytoplasmic staining and were obviously viable. Neutrophilic granules ordinarily are colored yellow, but the striking cytoplasmic staining appeared to be due to dye-containing vacuoles. The cytoplasm of mononuclear cells appeared to take up the dye also.

TABLE I. NEUTRAL RED STAINING OF LEUKOCYTES FROM NORMAL CONTROLS (NUMBER OF LEUKOCYTES STAINED PER 100 CELLS COUNTED)

	TIME IN HOURS																							
	0			1			1½			2			2½			3			3½			4		
PATIENT	C*	cC*	RW*	C	cC	RW	C	cC	RW	C	cC	RW	C	cC	RW	C	cC	RW	C	cC	RW	C	cC	RW
M. A.	0	2	0	0	0	0	2	1	1	2	2	3	3	1	0	3	1	4	5	6	4	11	16	9
A. B.	2	0	0	7	3	6	11	9	12	14	13	15	14	17	16	18	14	19	17	19	15	14	18	20
F. A.	0	1	0	4	3	3	2	3	5	7	8	10	11	9	10	13	14	18	16	16	19	15	17	20
J. C.	2	0	1	2	1	4	2	3	2	5	6	4	10	9	12	8	12	16	10	15	15	14	18	14
E. G.	0	2	0	2	1	3	5	4	4	7	9	11	13	13	14	11	14	19	13	16	18	15	17	22
B. S.	0	0	0	2	0	1	2	1	3	4	2	2	6	7	5	5	4	6	5	7	11	9	10	16
V. S.	0	0	1	5	4	7	7	8	6	10	8	11	9	12	10	14	14	15	17	16	19	19	17	18
Z. M.	2	0	0	6	8	16	9	11	12	11	13	12	10	14	11	16	14	17	12	23	19	18	20	25
E. E.	3	4	3	2	3	3	4	8	9	7	11	14	10	11	13	15	16	19	17	15	20	21	21	24
R. H.	1	0	2	3	2	5	2	4	7	6	8	5	10	13	16	11	14	14	18	12	15	18	19	22

*C — Control. cC — Control with Coca's solution. RW — Ragweed antigen (0.1 ml. of 1,000 PNU per milliliter) added.

TABLE II. NEUTRAL RED STAINING OF LEUKOCYTES FROM RAGWEED-SENSITIVE PERSONS (NUMBER OF LEUKOCYTES STAINED PER 100 CELLS COUNTED)

	HOUR OF OBSERVATION (AFTER ADDITION OF ANTIGEN)															
	0		1		1½		2		2½		3		3½		4	
PATIENT	C*	RW*	C	RW	C	RW	C	RW	C	RW	C	RW	C	RW	C	RW
F. C.	1	19	2	29	4	38	5	44	10	41	7	44	13	48	18	46
M. H.	1	3	4	13	12	16	17	23	27	44	25	47	28	47	32	49
M. W.	0	0	0	0	2	3	4	6	7	6	11	14	17	18	19	18
C. G.	0	1	2	4	2	5	4	17	9	21	12	28	16	31	19	36
L. M.	0	0	5	16	18	43	31	68	45	71	53	81	49	73	56	79
V. H.	0	0	4	12	9	31	21	41	34	56	40	54	42	57	49	59
M. J.	1	0	3	1	5	9	6	5	10	10	14	17	12	16	13	15
S. S.	2	0	1	4	3	7	6	12	10	26	17	23	19	27	24	32
M. G.	0	1	2	4	3	7	4	12	11	17	14	17	18	19	24	26
R. M.	1	3	3	12	7	20	8	24	11	25	17	26	14	23	19	28
M. E.	4	9	8	21	17	43	20	44	24	48	25	44	28	47	22	46
J. C.	0	0	3	7	4	10	9	13	14	19	15	18	11	17	18	18
S. R.	0	2	1	9	8	15	11	21	11	23	17	25	23	31	39	38
H. P.	0	1	3	7	5	5	11	13	17	27	20	39	24	37	31	36
W. H.	1	2	3	8	3	10	7	15	10	16	12	18	16	18	13	20
C. T.	0	0	2	13	2	21	6	25	9	26	12	24	19	30	18	26

*C — Control. RW — Blood with ragweed antigen (0.1 ml. of 1,000 PNU per milliliter added.

TABLE III. NEUTRAL RED STAINING OF LEUKOCYTES FROM RAGWEED-SENSITIVE

	TIME IN								
	0			1			1½		
SUBJECT	C*	10,000† RW*	1,000† RW	C	10,000† RW	1,000† RW	C	10,000† RW	1,000† RW
P. H.	0	0	1	3	8	7	5	6	5
L. M.	1	2	1	3	6	7	14	29	28
L. M.	1	4	3	4	13	14	5	17	17
W. H.	0	2	2	3	11	8	3	14	10
C. T.	2	0	1	4	11	10	3	14	16
N. O.	3	1	3	9	18	12	13	25	27
M. J.	3	1	3	4	6	3	7	6	8
C. G.	1	0	2	6	10	8	17	24	18
Mean	1.4	1.3	2	4.5	10.4	8.6	8.4	16.9	16.1
S.D. ±	1.2	1.4	.9	2.1	3.9	3.4	5.5	8.5	
P						$< .005$			$< .005$

*C — Control. RW — Ragweed.
†PNU per milliliter.

The results obtained from studies in ten normal controls are shown in Table I. No difference (significant differences were those when $P < 0.005$) in the percentage of stained cells was found, whether Coca's solution alone was added to the blood or whether nothing was added.

Table II shows the percentage of stained leukocytes in the blood of ragweed-sensitive individuals to which nothing was added, and the per cent stained in blood to which 0.10 ml. of 1,000 PNU per milliliter of ragweed antigen was added. The number of stained leukocytes in 100 white blood cells was significantly increased ($P < 0.005$) when the antigen was added, the difference being greater after one and one-half to two and one-half hours. There was no significant difference in samples treated by the addition of 0.10 ml. of 1,000 PNU per milliliter of ragweed extract, or 0.10 ml. of 10,000 PNU per milliliter as shown in Table III. Surprisingly, the percentage of stained leukocytes was considerably lower in samples from nonallergic individuals when compared with the percentage of stained leukocytes from ragweed-sensitive individuals, whether exposed to antigen or not.

Although not shown here in tabular form, when 10 mg. of EDTA was added to a blood sample, no significant effect upon the staining of ragweed-exposed leukocytes was encountered.

In the blood of four individuals, histamine added to a final concentration of 10 μg per liter did not produce any significant change, whereas the addition of histamine in larger doses (60 μg per liter) caused a remarkable increase in the number of stained cells in both the control and ragweed-treated blood. These findings were essentially the same whether the blood was incubated at 37° C. or kept at room temperature.

DISCUSSION

As seen in Table II, the addition of the antigen to the blood of ragweed-sensitive individuals produces a significant increase in the number of stained leukocytes when compared to the control blood of the same individual to which no antigen was added. Although the complete tabulation of information is

SUBJECTS (NUMBER OF LEUKOCYTES STAINED PER 100 CELLS COUNTED)

HOURS											
2			2½			3			3½		
C	10,000† RW	1,000† RW	C	10,000† RW	1,000† RW	C	10,000† RW	1,000† RW	C	10,000† RW	1,000† RW
11	13	13	17	28	27	20	37	39	24	38	37
28	47	49	41	68	63	46	71	72	58	83	82
9	20	18	11	23	20	14	21	22	16	22	19
7	18	15	10	17	16	12	15	18	16	19	18
7	23	21	10	22	22	10	19	21	17	23	23
16	33	38	17	32	33	18	33	29	23	36	39
7	10	9	13	15	12	18	17	20	20	21	20
19	28	33	22	29	30	21	32	33	23	35	34
13	24	24.5	17.6	29.3	27.9	19.9	30.6	31.8	24.6	34.6	34.0
7.5	11.9	10.9	10.3	16.7	15.8	11.2	18.3	17.8	14.0	21	21.2
		$< .005$			$< .005$			$< .005$			$< .005$

not presented here, there appears to be some correlation between the severity of the clinical manifestations, degree of intradermal skin reaction, and the percentage of stained leukocytes, although two subjects (M. J. and J. C.) with histories of severe sensitivity did not show any significant increase in the percentage of stained cells. There was no apparent difference of results in patients who received hyposensitizing therapy and those who did not.

Cytoplasmic staining of leukocytes by neutral red has been termed "vacuolar staining"[7] and is commonly observed with the usual supravital techniques. The appearance of the living leukocyte, with red or brown vacuoles in the cytoplasm, is quite in contrast with that of the dead cell in which the vacuoles disappear and the nucleus is stained. Little is known of the factors which control the propensity of leukocytes for vacuolar staining. Admittedly it is a hazardous presumption, but factors influencing the formation of neutral red vacuoles may be related to those affecting pinocytosis and phagocytosis, which have been shown to be controlled by factors including temperature, pH, tonicity, oxygen tension, and hormonal influences.

In our studies, as in those of others who have studied various effects of ragweed antigen upon leukocytes from an individual sensitive to ragweed, there is no means of controlling these factors beyond a uniformity of method in carrying out each observation. The results, therefore, must be evaluated with even more than the usual reserve accorded this type of experimentation.

It appears that, even in the absence of ragweed antigen, the cytoplasm of ragweed-sensitive leukocytes shows an increased amount of vacuolar staining. This activity is further increased in the presence of minute amounts of ragweed antigen.

One purpose of these observations was to determine whether the increased staining of leukocytes exposed to antigen was associated with a release of histamine. Since EDTA inhibits blood histamine release by chelation of divalent cations, the effect of the presence of EDTA was studied. EDTA had no effect upon the reaction, indicating that the increased uptake of dye is not associated with histamine release. Because histamine release proceeds actively

at 37° C., but more slowly at room temperature,[9] a comparison of the two was made and no difference was found. However, there was a significant difference when compared to samples to which 60 μg per liter of histamine was added. This might suggest that histamine, when present in sufficient amount, may alter the cell membrane's permeability to allow the staining of the cytoplasm.

CONCLUSION

The addition of ragweed antigen to the blood of ragweed-sensitive individuals in vitro produces a significant increase in the number of neutral red-stained leukocytes when compared with the number stained when no antigen is added.

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DELAYED AND IMMEDIATE SKIN REACTIVITY IN MAN AFTER THE INJECTION OF ANTIGEN IN EMULSION

Cell Transfer of the Delayed Sensitivity

Robert J. Becker, M.D., Dale B. Sparks, M.D., Samuel M. Feinberg, M.D., Roy Patterson, M.D., Jacob J. Pruzansky, Ph.D., and Alan R. Feinberg, M.D., Chicago, Ill.

In 1959 in the course of investigations on absorption and immunologic aspects of ragweed extract injected in Arlacel A–Drakeol emulsions it was found[1] that five out of six nonatopic subjects developed a delayed skin reactivity to ragweed antigen. The subject who had the strongest skin reactivity had marked inflammation of his arm at the site of the repository injection which later fluctuated and drained as a sterile abscess. In an effort to confirm these findings and to extend these observations, fifteen nonatopic volunteer subjects from a penal institution were selected for further study in 1960.

PROCEDURE

The subjects selected gave a negative personal and family history of atopic manifestations, as well as negative intradermal tests to mixed giant and short ragweed 1:1,000 (1,000 pollen units or 390 protein nitrogen units per milliliter). On July 26, 1960, each subject received an intramuscular injection of 0.5 ml. in each arm of an emulsion containing a mixture of 10,000 pollen units of giant and short ragweed extracts (3,900 protein nitrogen units per milliliter of emulsion). The emulsion mixture was prepared by adding 1 part of an equal mixture of giant and short ragweed extract containing 30,000 pollen units to 2 parts of an Arlacel A–Drakeol solution (1:9). The mixture was emulsified by passing through a double hub 18-gauge needle attached to two 10 ml. Luer-Lok syringes. The ragweed extract used in the emulsion was extracted with Coca-merthiolate solution and was the same as that used for the preliminary and subsequent skin tests, and supplied for the nationwide collaborative ragweed repository study by the Committee on Specific Therapy of the American Academy of Allergy.[2]

Beginning four weeks after the emulsion injection, intradermal tests were made with pollen extracts containing 10, 100, and 1,000 pollen units per milliliter. These tests were repeated at intervals for several months. Thirteen out

From the Allergy Unit, Department of Medicine, Northwestern University Medical School.
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of the fifteen subjects showed a skin reactivity on the first test. In nine, the induced reactivity was of the delayed type and consisted of a papule with erythema beginning six to twenty-four hours after the injection, persisting to some extent for days and which, in the more extreme instance, resulted in vesiculation and a superficial necrotic lesion (Fig. 1). The specificity of the reactions was indicated by the inability of this ragweed preparation to react in other nonallergic individuals. In seven, the reaction was of the immediate wheal and erythema type, associated with itching and disappearance in one to several hours. Three of the nine delayed reactors had this immediate type reaction.

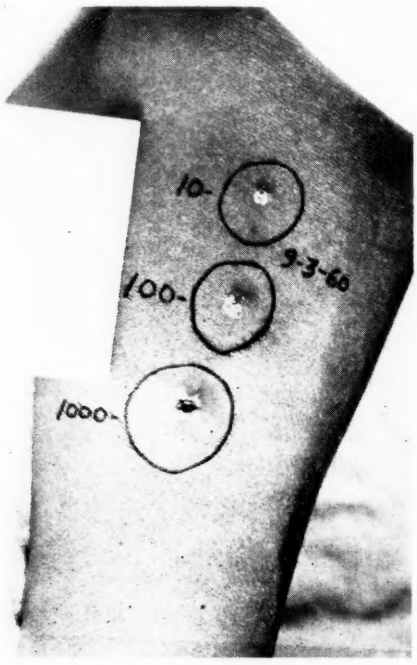

Fig. 1.—Vesiculation and superficial necrotic lesions in the Subject J. O. twenty-four hours after an intradermal injection of ragweed.

At the time of this writing, six months after the repository injection and five months after the original testing, one subject changed from an immediate into a delayed type reactor, one added a delayed reactivity to his immediate type, and a third added an immediate reactivity to his delayed type. Two of the original nonatopic individuals who received ragweed emulsion and did not develop delayed or immediate sensitivity were given a cell transfer and converted to a positive delayed reaction when tested six days later.*

Those subjects in whom a delayed hypersensitivity type of skin test was found had been observed to develop swollen and tender arms at the site of the repository injection, beginning on the average about ten days after the injection. Three of these developed distinct tense masses at the sites of injection and one

*One of the nonatopic subjects of the series described a year ago, who had developed a strong delayed skin reactivity to ragweed and a sterile abscess after a repository injection, was retested eighteen months after his initial sensitivity, and still showed a marked skin reactivity to ragweed 1:10,000.

appeared to be fluctuating, but did not drain. The latter subject complained of malaise and weakness for eight weeks after the injection and had limitation of movement of his arms for several weeks. The muscle swelling and limitation in motion slowly decreased and by the end of seven months only a firm 3 cm. mass remains. None of the subjects developed clinical signs of symptoms referable to pollenosis during the ragweed season.

Conjunctival tests were made with a small speck of whole ragweed pollen, using other pollen as a control test in the other eye and in the eyes of non-allergic individuals. Of the six subjects with immediate skin reactivity in whom conjunctival pollen tests were done, three gave a typically positive reaction. Passive transfer tests were done with the sera of six subjects with immediate type skin reactivity. Only one gave a definitely positive PK reaction.

At the same penal institution, twenty-seven patients with ragweed hay fever and with positive skin tests to ragweed extract received repository ragweed therapy in doses varying from 100 to 10,000 pollen units. None of these developed a delayed skin reaction to pollen extract and none of them experienced a delayed inflammation at the site of the repository injection. Eleven patients received the emulsion without the ragweed extract as part of a double-blind therapy study. None of these developed any type of delayed inflammatory response.

In the fifteen experimental subjects, the post-treatment sera failed to show precipitin reactions with ragweed extracts on gel diffusion, and were negative in attempts to produce passive cutaneous anaphylaxis in the guinea pig. Tanned cell hemagglutination studies* on the sera showed the following: The subject (J. O.), previously described as having had the most exquisite delayed hypersensitivity, showed a titer of 1:2,560. Twelve had titers ranging from 1:20 to 1:80. Two were negative, as were control sera. Aside from the one who had the very high titer, there was no obvious relationship between the titer, or absence or presence of hemagglutination, and the absence or presence of immediate or delayed skin reactivity.

The findings just described are summarized in Table I.

CELL TRANSFER

As shown by Landsteiner[3] and Chase[4] in animals and Lawrence and his associates[5-8] in man, delayed hypersensitivity of certain types can be transferred by leukocytes either obtained from inflammatory exudates or from peripheral blood. Because the genesis of the type of delayed appearing hypersensitivity described by us was different from the conventional (microbial), it was important to determine whether it possessed the same characteristic of cell transfer. Each of three subjects with delayed hypersensitivity to ragweed contributed 250 ml. of blood. The white cells were harvested according to a modified method of Gold and Cole,[9] using polyvinylpyrrolidone and heparin. The washed leukocytes were injected intradermally and subcutaneously into 2 sites.

Tests were made at the sites of the intradermal transfer and at distant sites with ragweed extract six days after the transfer. There was no immediate

*Done by Dr. Carl E. Arbesman, Buffalo, N. Y.

TABLE I. RESULTS OF INJECTION OF 10,000 POLLEN UNITS OF RAGWEED EMULSION INTO NONATOPIC SUBJECTS ON JULY 26, 1960

SUBJECT	FIRST POST-REPOSITORY TESTS (AUG. 26, 1960)				FINAL TESTS (JAN. 18, 1961)		INTRADERMAL TESTS WITH RAGWEED 1,000 μ/ML. AFTER SERUM TRANSFER	
	DELAYED WEAKEST DILUTION POSITIVE POLLEN (μ/ML.)	IMMEDIATE WEAKEST DILUTION POSITIVE POLLEN (μ/ML.)	CONJUNCTIVAL TEST WITH WHOLE POLLEN (OCT. 18, 1960)	HEMAGGLUTINATION TITER	DELAYED WEAKEST DILUTION POSITIVE POLLEN (μ/ML.)	IMMEDIATE WEAKEST DILUTION POSITIVE POLLEN (μ/ML.)	IMMEDIATE	DELAYED
1. H. F.	Neg.	1,000	Neg.	1:20	1,000	1,000	Neg.	Neg.
2. R. K.	Neg.	1,000	Neg.	1:80	1,000	Neg.	Neg.	Neg.
3. C. R.	Neg.	1,000	Neg.	1:80	—	—	Pos.	Pos.
4. J. A.	10	1,000	Pos.	1:40	1,000	100	—	—
5. S. V.	1,000	100	Pos.	0	1,000	1,000	Neg.	Neg.
6. J. O.	10	Neg.	—	1:2,560	10	100	Neg.	Neg.
7. S. D.	Neg.	1,000	Pos.	1:80	Neg.	1,000	—	—
8. R. H.	1,000	1,000	—	1:80	—	—	Neg.	Neg.
9. L. B.	1,000	Neg.	—	1:80	1,000	Neg.	—	—
10. J. H.	1,000	Neg.	—	0	1,000	Neg.	—	—
11. D. M.	1,000	Neg.	—	1:40	1,000	Neg.	—	—
12. H. K.	1,000	Neg.	—	1:80	1,000	Neg.	—	—
13. G. W.	1,000	Neg.	—	1:40	1,000	Neg.	—	—
14. C. Mc.	Neg.	Neg.	—	1:80	1,000*	Neg.	—	—
15. F. Z.	Neg.	Neg.	—	1:20	1,000†	Neg.	—	—

*WBC transfer from J. O. with conversion of skin test local and general when tested six days later.
*WBC transfer from S. V. with conversion of skin test local and general when tested six days later.

reaction, but beginning about six hours later a delayed type skin reaction occurred which was comparable in appearance to that previously obtained in the donor. Four positive cell transfers have been made from the three donors. Two of the recipients (C. Mc. and F. Z.) were in the original group of fifteen nonatopic subjects. They were tested and found to be negative prior to cell transfer, but gave a positive reaction when tested six days later. The other two recipients were nonatopic individuals and not sensitive to ragweed when tested prior to cell transfer. Transfer of the serum from the donor did not transfer delayed hypersensitivity.

Fig. 2.—Photomicrograph of a skin punch biopsy made twenty-four hours after the intradermal injection of ragweed which shows perivascular infiltration with round cells. (January, 1961.)

A punch biopsy made twenty-four hours after the intradermal injection of the antigen at a site distant from the site of the transferred leukocytes showed a perivascular infiltration with round cells (Fig. 2). This finding is compatible with, but not diagnostic of, a delayed type of inflammatory response.

DISCUSSION

The clinical implications of the findings described here may be of importance. It is obvious that at least in nonatopic individuals, who are not allergic to ragweed, hypersensitivity is produced to ragweed antigen injected in Arlacel A–Drakeol emulsion. Thus far the immediate reacting antibodies

have been of moderate degree and unlikely to produce clinical pollenosis. Although the immediate reactivity has not disappeared after several months, neither has it increased. It is possible also that, in those who are allergic to ragweed, the injection of repository ragweed might actually increase the sensitizing antibody, although increased blocking antibody or other protective mechanisms might sufficiently neutralize and exceed the effect. If an increase in immediate reacting antibody in the amounts we have found is obtained in the allergic person, it would probably be insufficient to be titrated as a change in the already large titer of reagin. It is difficult to understand why in the series of induced delayed ragweed sensitivity last year we did not encounter any subjects who developed immediate reactivity.

The production of delayed reactivity may be of greater clinical importance. In the first place, as noted in this series and in those of a year ago, it is prone to manifest itself in severe prolonged inflammation and abscess formation at the site of injection. The development of delayed reactivity may also imply the production of a delayed type of inflammation on natural exposure to the antigen (for example, eye inflammation during the ragweed season). Thus far there has been no evidence of that. It is conceivable also, but even more highly speculative, that the repository therapy may in some way set off other types of allergic responses in viscera, perhaps unrelated to the original antigen. Until more extended investigations are made the inference should be that an antigen should not be injected in emulsion form unless the physician is certain that the patient is allergic to it. This implies the necessity for more careful appraisal of what is injected. A questionable dust reaction (which could be nonspecific), the use of mixtures of molds to some of which the patient may not be allergic, or the use of a variety of tree pollen extracts in a "tree" mixture could lead to trouble. There is reason to think that at least in some of the cases in which sterile abscess has occurred in the past the presence of a previous allergy to that antigen was questionable. Since any extract (ragweed pollen, for example) has a number of antigens and the patient may not be allergic to some of them, the likelihood of inducing new allergies must even be considered as a possibility in the average case. It is possible that the persistence of delayed sensitivity is to some extent influenced by the repeated intradermal tests.

The basic, and perhaps the practical, question is: Why does the nonallergic person develop a sensitivity from ragweed repository injection, while the person allergic to ragweed does not? Is this on a genetic or constitutional basis? Or does the lack of an immediate antigen-antibody reaction in some way favor the production of delayed sensitivity? Is this a peculiar property of the ragweed antigen? It has been also suggested that Arlacel A might be responsible while another emulsifying agent might not produce such effects. Investigations are under way in an attempt to answer these and other pertinent questions relative to this phenomenon of induced sensitivity.

This finding has also presented us with a tool for studying delayed sensitivity, the long-debated question of conversion of one type of sensitivity into the other, and a number of other aspects of sensitivity, particularly of the delayed type.

SUMMARY

In fifteen nonallergic subjects receiving ragweed in repository form, seven developed immediate skin reactivity, nine delayed reactivity, and three both. After six months all still react.

In those developing delayed skin reactivity there was an inflammatory swelling of varying degree at the site of the repository injection, beginning several days later and lasting for several weeks.

The delayed reactivity to ragweed was transferred locally and systemically by the injection of peripheral leukocytes from three subjects in whom delayed sensitivity had been induced by an injection of ragweed emulsion.

Punch biopsy from the delayed reaction produced in the recipient showed histologic changes characteristic (but not pathognomonic) for a delayed hypersensitivity response.

The findings point to the advisability of confining repository treatment to antigens to which the patient is known to be sensitive.

This investigative model offers an opportunity for basic studies on delayed hypersensitivity and the relationship between the delayed and immediate type.

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A FLUOROMETRIC METHOD TO DETERMINE LEVELS OF HISTAMINE IN HUMAN PLASMA

Joseph W. Noah, M.D., and Alta Brand, St. Louis, Mo.

THE CONCEPT of the importance of histamine as a chemical mediator in atopic disease has stimulated those who search for increasingly sensitive, yet simpler, methods of measuring this compound. This report concerns a fluorometric method devised by Shore, Burkhalter, and Cohn,[1] and modifications which render it more suitable for the determination of histamine levels in human plasma. In the past, determinations of plasma histamine have largely been carried out by either the bio-assay method, measuring the contractile effect of histamine on guinea pig ileum, or by a spectrophotometric method which measures the monodinitrophenyl derivative of histamine's reaction with 2, 4-dinitrofluorobenzene under fixed conditions of pH, temperature, and dinitrofluorobenzene concentration.[2, 3] The wide application of these two methods has resulted from their greater sensitivity and reproducibility, as compared with other procedures.

No method of determining histamine in biologic substances utilizes the absolute measurement of histamine, in the manner in which the chemist applies it in isolating a substance, weighing it, and identifying the compound by determination of crystalline structure, melting point, and solubility. All methods of histamine determination, including the fluorometric, are plagued by interfering substances which have to be removed before the histamine present can be measured accurately. Utilizing the fluorescing property of a condensation product of histamine with o-phthalaldehyde, the fluorometric method has the advantage not only of greater sensitivity but also of a simpler, faster means of separating histamine from interfering materials. With most tissues, Shore and co-workers have been able to demonstrate very satisfactory results with their method. In making determinations of human plasma histamine, however, our results have consistently shown higher values with the fluorometric method than with the dinitrofluorobenzene method of Lowry and his associates.[2] The interfering substance or substances present detract at times from the ability of the method to detect changes in levels of plasma histamine. When more accurate values are required, they can be achieved by the use of decalso columns with the fluorometric method.

From the Department of Medicine, Washington University School of Medicine, St. Louis, Mo.

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METHOD

Comparison of this description with that of Shore and his group will reveal that the modification to permit the use of small volumes of plasma is largely a scaling-down of quantities of reagents involved.* Because of the ubiquity of interfering fluorophores, all reagents used must be of the highest obtainable purity. Distilled water must be triple-distilled in glass. Exposure of any of the materials involved to rubber, plastics, or silicones may produce erratic results. All glassware, after conventional cleaning with detergent and water, is boiled in 50 per cent nitric acid and further cleansed with distilled water. After four to seven days, blank readings usually increase and new reagent solutions must be prepared.

In our studies, a sample of blood, withdrawn from the antecubital vein of suitable subjects into syringes wet on the interior surfaces with, but containing no excess of, sodium heparin, was divided into aliquots of approximately 2 ml. To obtain histamine release from the blood of ragweed-sensitive subjects, 20 μl of 10,000 P.N.U. per milliliter of ragweed extract was delivered by a constriction micropipette into duplicate tubes containing 2 ml. blood samples, while nothing was added to another pair of aliquots which served as controls. After incubation in a water bath at 37° C. for twenty minutes, the tubes were centrifuged at 3,500 r.p.m. for thirty minutes to pack the cells, and thereupon, 1 ml. samples of plasma were withdrawn from each tube. Plasma control tubes were incubated for the same interval. To each plasma sample was added by micropipette 112 μl† of 50 per cent redistilled trichloroacetic acid (TCA). After sufficient buzzing to insure thorough mixing, the mixture was allowed to stand for thirty minutes, with a short period of buzzing fifteen minutes after adding the acid and again at the termination of the thirty minute period. The mixture was centrifuged at 3,500 r.p.m. for forty minutes. Five hundred microliters of the clear supernatant liquid was then removed from the plasma–TCA mixture, the remainder of which was discarded. The plasma supernates, along with duplicate reagent blanks, consisting of 500 μl of 5 per cent TCA, and histamine standards, containing 0.002, and 0.01, and 0.015 μg of histamine base in 500 μl of 5 per cent TCA, were treated in the following manner.

To each tube were added 0.2 Gm. NaCl, 60 μl of 5 N NaOH, and 1.25 ml. of n-butyl alcohol. The addition of each material was followed by thorough buzzing. After standing, to permit separation of the aqueous and alcoholic layers, the aqueous layer was removed and discarded. The butanol portion was washed twice with 1.0 ml. amounts of 0.1 N NaOH saturated with NaCl. After the second NaOH–NaCl wash, 1.0 ml. of the butanol layer was added to 1.5 ml. of n-heptane and 380 μl of 0.1 N HCl. After buzzing and standing long enough to permit separation of layers, 300 μl of the acid layer was withdrawn, and to this were added 60 μl of 1 N NaOH, and after mixing, 14 μl of 0.1 per cent

*This is an adaptation of Dr. Helen Graham's modification of the fluorometric method. Comparison of estimates of tissue histamine made by this adaptation and by the dinitrofluorobenzene method will be published shortly by Dr. Graham.

†This quantity was determined by the capacity of the particular micropipette used. In this and other reagent amounts, the precise amount is determined by micropipettes available.

o-phthalaldehyde (OPT). After standing at room temperature for four minutes, 25 μl of 3 N HCl was added. Within thirty minutes, this mixture was placed in a small cuvette and the intensity of fluorescence determined in a fluorometer.*

The concentration of histamine was calculated as follows:

$$\frac{PV + TCA}{SN} \times 1.1\dagger \times \frac{U}{S} \times \mu g \text{ of histamine} \times \frac{1{,}000}{\text{ml. plasma}} = \mu g \text{ of histamine/L., plasma}$$

where PV = water volume of plasma, which is actual volume of plasma × 0.94; U = reading of unknown; S = reading of standard; TCA = added volume of TCA in ml.; SN = volume of supernate used on column or with butanol.

As will be demonstrated, interfering substances were better removed by the use, in addition to the above procedures, of the decalso column, as described by Lowry and associates.[2] To do this, the TCA supernate was, after addition of 4 M sodium acetate to the amount of 1/10 of the volume of the supernate (enough to give a pH of 3.4), allowed to run through a decalso column, and the adsorbed histamine then eluted with 40 per cent KBr in a hot box at 80° C. The KBr solution containing the histamine was treated in precisely the same manner as the TCA supernate ordinarily is—that is, NaCl, NaOH, and n-butanol are added as described above, and the remaining steps of the fluorometric technique carried out.

RESULTS

With precautions to avoid damage to the cells in collecting and handling the whole blood, and to avoid introducing white cells into a plasma sample as it is secured, the average level of true histamine in human plasma has been found to be 2.6 mg. per liter when the DNFB method is used.[2] This finding has been confirmed by our own experience with the DNFB method.

Levels of human plasma histamine by the OPT method in our hands are considerably higher. The average histamine value of 34 plasma samples was found to be 13.6 μg per liter. The possibility that an interfering substance or substances produced the high values was enhanced by the discovery that the addition of histidine to plasma produces an apparent increase in levels of plasma histamine. The addition of a second wash of the butanol containing histamine assists in removing additional amounts of histidine and other substances which react with OPT, but not in amount great enough to be of decisive value. Additional washings also reduce the amount of histamine left in the butanol.

A number of histamine release studies, performed on the blood of nonallergic controls and ragweed-sensitive subjects, are shown in Table I. To reduce the amount of interfering substances, the use of the decalso column has

*A Turner Fluorometer, Model 110, was used for these determinations. To read samples of 250μl volume, a special adapter, generously supplied by Dr. Robert E. Phillips of G. K. Turner Associates, Palo Alto, Calif., was installed in place of the regular cuvette holder. This permits the use of 6 mm. by 50 mm. standard Pyrex tubes as cuvettes. The primary filter used (color specification #7-60) and the secondary filter (color specification #2-A) are those routinely supplied with this instrument.

†Factor used only with decalso columns, to correct for added sodium acetate.

been found helpful. An average of plasma controls which have been done by both methods shows an average plasma histamine level of 15.7 μg per liter without the use of decalso columns, 3.03 μg per liter with columns. The latter value is in excellent agreement with that found by Lowry and co-workers,[2] especially when one considers that the OPT determinations were on samples subjected to the additional manipulation of incubation at 37° C.

TABLE I. PLASMA HISTAMINE RELEASE VALUES BY OPT METHOD IN NONALLERGIC AND RAGWEED-SENSITIVE SUBJECTS*

PATIENT		WITH COLUMN	WITHOUT COLUMN
J. N.	(C)†	2	17
	(R)†	5	17
A. B.	(C)	3	18
	(R)	5	20
J. I.	(C)	5	15
	(R)	5	22
R. P.	(C)	1	13
	(R)	1	14
J. B.	(C)	2	13
	(R)	7	25
R. Y.	(C)	1	25
	(R)	21	28
M. F.	(C)	2	22
	(R)	31	41
C. G.	(C)	1	17
	(R)	60	51
C. S.	(C)	4	8
	(R)	11	21
E. K.	(C)	9	12
	(R)	27	61
J. U.	(C)	5	13
	(R)	59	71
W. N.	(C)	5	15
	(R)	52	64

*Figures refer to micrograms of histamine per liter.
†(C) = Control; (R) = Ragweed.

DISCUSSION

Findings obtained by the OPT technique concerning histamine release do not conflict with those made previously using the DNFB method. The value of the OPT method lies in its greater simplicity and rapidity, but largely in the relatively small blood sample which is needed for a single determination. As shown, at present it seems practical to use plasma samples of 1.0 ml., although we have used plasma samples of 360 μl volume with good, but more erratic, results. With improved equipment, blood histamine release studies can probably be carried out on human blood with samples of 1.0 ml. or less. This places the technique within the realm of practical clinical application.

Although apparently not true for other biologic substances tested,[1] interfering substances, including histidine, are present in human plasma in amounts which may, at times, interfere with histamine release studies. The additional step of adsorbing the histamine on a decalso column and eluting it therefrom will largely remove the contaminants.

CONCLUSION

The OPT method offers an improved means of studying human blood histamine release in small blood samples.

The help of Dr. Helen Graham, Department of Pharmacology, Washington University School of Medicine, is gratefully acknowledged. Dr. Graham not only gave us valuable counsel but also permitted the use of equipment and material in her laboratory.

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MAINTAINING RAGWEED CULTURES

Willard W. Payne and Walter F. Kleinschmidt, Ann Arbor, Mich.

EXPERIMENTS being carried out on the nature of the ragweed plant and hay fever have made it necessary to grow many specimens in the greenhouse and garden. These ragweed cultures provide a continuous supply of material for botanical studies and implement physiologic research on growth and pollen production by the ragweeds. Specimens are also produced for studying the dissemination of ragweed pollen outside the natural ragweed season, and fresh pollen is made regularly available for immunologic and clinical studies.

Techniques for maintaining an adequate supply of viable ragweed seeds available throughout the year to the experimenter were worked out in the growing season and winter of 1959-1960. As research workers in allergy and related fields may be interested in growing ragweed plants for experimentation, our after-ripening technique is described later, along with methods of collecting and storing fruits, culturing plants, and collecting pollen.

COLLECTING RAGWEED FRUITS

Because of their adaptation for wind pollination rather than insect pollination, ragweeds have evolved a structure and appearance quite different from most other members of the composite family, the botanical family to which they belong, along with such familiar plants as chrysanthemums, sunflowers, and dandelions. Flower colors and forms that function to attract insects have been lost. Individual flowers are much reduced and the functions of pollen and fruit production have been relegated to different flowers on different parts of the plant. A normal ragweed plant, therefore, bears heads or flower clusters of two kinds: pollen-producing or male flowers are clustered in heads which are borne in spikes at the tips of the main stem and branches; seed-producing or female flowers are borne singly in modified heads located in the axils of leaves and bracts below the male flower spikes. Fig. 1, *A-C*, shows male and female heads and their usual arrangement on a branch of giant ragweed.

The seeds are produced in the fall. Each seed is enclosed in a dry fruit surrounded by the fused, hardened bracts (involucre) of the female head.

From the Department of Botany and Botanical Gardens, University of Michigan, Ann Arbor, Mich.

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Typical fruits of the more abundant ragweed types are shown in Fig. 1, *D-H*. These are collected in September or October, after they have matured, by grasping the stem a few inches below the tip and stripping it of leaves, fruits, and spikes. After drying for a few days, the fruits are freed from the unwanted elements by rubbing all of the material between the hands. Light gloves are usually worn to protect the hands from the hard, sharp spines. By carefully pouring the mixture of fruits and vegetative fragments from one pan to

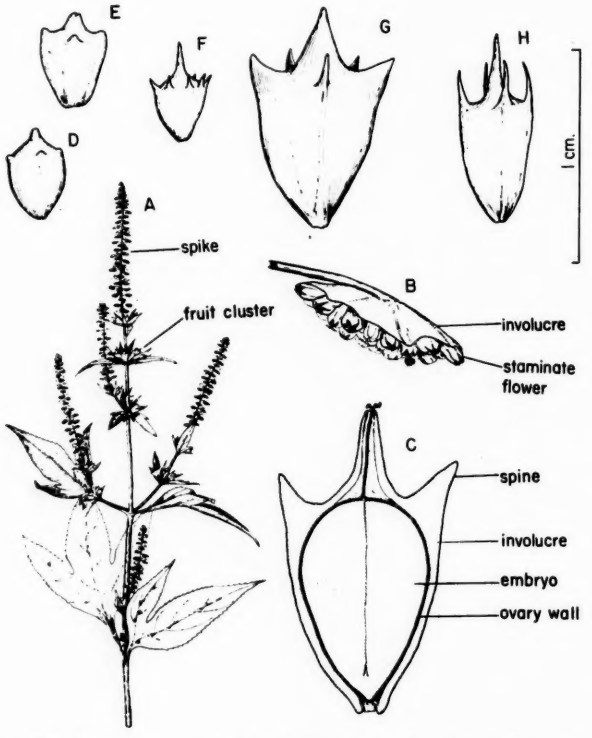

Fig. 1.—Ragweed inflorescence and fruit types. *A*, Flowering branch of giant ragweed *Ambrosia trifida*, showing the position of male and female heads. *B*, Enlargement of male head. *C*, Longitudinal section of a mature fruit. *D*, Fruit of eastern perennial ragweed, *A. coronopifolia* T. & G. *E*, Fruit of western perennial ragweed, *A. psilostachya* DC. *F*, Fruit of common ragweed, *A. artemisiifolia* L. *G*, Fruit of giant ragweed, *A. trifida* L. *H*, Fruit of southern ragweed, *A. bidentata* Michx.

another in front of a fan, the fruits are winnowed out. Lighter debris, consisting of pieces of leaves, male heads and stems, is blown away while the heavier fruits fall through the air stream. Empty fruits, those which develop with no seeds enclosed, are light and are blown away with the other debris.

AFTER-RIPENING*

Ragweed fruits are dormant when they are collected from the plant. This dormancy is broken by the process termed after-ripening. After-ripening

*Limited quantities of fruits which have been after-ripened in the manner described will be available during the growing seasons of 1961 and 1962. Fruits of the following species can be obtained by addressing inquiries to the senior author: Low or common ragweed, *Ambrosia artemisiifolia* L.; giant ragweed, *A. trifida* L.; eastern perennial ragweed, *A. coronopifolia* T. & G.; southern ragweed, *A. bidentata* Michx.

is accomplished by allowing the embryos in the seeds to undergo a period of rest during which the conditions required for germination are not present. In northern latitudes it is done by submitting them to a period of low temperatures while supplying them with sufficient water and oxygen. Under natural conditions, the occurrence of winter accomplishes after-ripening. The artificial technique given below is designed to simulate natural environmental conditions as closely as possible.

1. The fruits are placed in cloth bags. Inexpensive cotton broadcloth or similar material made from a blend of cotton and synthetic fibers is used. Old or worn material tends to decompose during the winter treatment and is not satisfactory. The size of the bags is varied to suit the number of fruits to be treated, but the largest bags we use are approximately eight by twelve inches. Plastic bags or jars do not permit development of the proper moisture and oxygen conditions and their use often results in loss of the seed through death of the embryos or failure to break dormancy. If several kinds of fruits are gathered, necessary data is written on the bags with a laundry pen of the ball-point type. Such a pen does not catch in the material as the ordinary quill-type laundry marker does.

Care is taken not to put too many fruits in one bag. Respiration of crowded seeds may raise the temperature in the bag and inhibit after-ripening or promote germination in the bag. Crowding also restricts the oxygen supply by keeping the fruits sodden.

2. The fruits are soaked in lukewarm water for an hour. Since after-ripening is successful and uniform only if the embryos are saturated with water,[1] this is a very necessary step.

3. Approximately an inch of washed sand is placed in a wooden flat and the bags are spread on the sand. The layer of fruits is distributed evenly in each bag and the bags are then covered with another inch of washed sand. It is important that clean sand be used, as the movement of water and gases may be restricted if the sand contains too much clay or humus. At our Botanical Gardens a commercially available type known as "fine, washed, plastering sand" is used, but any clean sand is satisfactory.

4. The flats are sprinkled well with water and placed outside where they will not be disturbed. Since after-ripening requires approximately three months, this should be done by late November or early December. If done too early, however, mice or other rodents may be attracted to these handy caches of food and may eat or carry away most of the fruits. If rodents are abundant the flats can be protected by covering them with screens. The flats should not be covered with boards or other impermeable materials which will shield them from snow or frost. A shallow, open cold-frame is ideal.

5. The flats are brought inside in the early spring while the seeds and sand are still frozen. At our location this is late February or early March. Seeds may be germinated immediately or stored for future use. The after-ripened fruits are stored in two ways: (a) The frozen fruits are left in their after-ripening bags and placed in a freezer. Seeds stored in this way in our laboratories have remained viable for nearly a year. When plants are needed, a

quantity of fruits is removed from the bag, thawed, and sowed immediately. (b) The fruits are removed from the bag, spread in a cool place and air-dried as quickly as possible. Two days is usually adequate. Dried fruits are stored in loosely covered jars which allow circulation of oxygen between the air of the jar and the outer atmosphere. Seeds stored thus give us approximately 70 per cent germination ten months from the date of drying. Although no marked decrease in viability had been noted at the time this article was prepared, the seeds were expected to return eventually to a dormant condition. For this reason it is recommended that a new supply of fruits be grown and after-ripened annually to maintain a reliable source of viable seeds.

CULTURE OF PLANTS FOR POLLEN PRODUCTION

Three to four months before mature plants or fresh pollen are needed, a quantity of fruits is planted in a pot filled with light garden soil. The fruits are pressed firmly into the surface of the soil and covered with a thin layer of dry sand. The pot is then watered generously and placed in a cool greenhouse until germination occurs. The pot is covered with a pane of glass to prevent drying of the soil before the seedlings have developed.

The seedlings are potted off into small pots (two- or three-inch) as soon as they have emerged. If delayed too long the risk of loss of the seedlings through the activities of damping-off fungi is increased. After approximately a month the plants are potted in five- or six-inch pots.

Under a regime of normal day lengths, that is, the comparatively long days of spring and summer, the plants reach the pollen-producing stage in approximately three months. As each male head contains numerous flowers (Fig. 1, *B*) which mature in a gradual succession, the period of pollen production of a given head may extend over three or four weeks. A spike will produce the greatest quantity of pollen daily approximately a week to a week and one half after the initial flowering. Since spikes of the lower branches mature later than the terminal spikes, the period of pollen production for a given plant may be a month or more. Best results are obtained over prolonged periods by staggering plantings of fruits a week apart so that only the best producers need be used during the period for which pollen is needed.

The short days of winter cause the plants to mature when young and produce but few male heads or none. This phenomenon has been investigated by Jones[2, 3] and has been amply demonstrated in our greenhouses. Nearly normal plants can be grown if incandescent or fluorescent lights are used to extend the day length to twelve hours. In general, however, satisfactory pollen production is difficult to obtain in November, December, and January.

COLLECTING THE POLLEN

Pollen can be gathered in a number of ways. As the pollen is shed shortly after dawn, it must be collected by eight or nine o'clock in the morning. If many plants are grown, large quantities of pollen can be collected with a vacuum pump to which is attached a Buchner-type funnel with a fritted disc.

The funnel is run up and down the side of the spike while the spike is gently agitated. A few days' observation of the producing plants will reveal the best time for this operation. A somewhat more efficient but more painstaking method is to tap the spikes over waxed paper, allowing the pollen to fall onto the paper. The most efficient technique for gathering pollen is to allow the spikes to shed their pollen on paper after they have been removed from the plant.[4] For this last technique a quantity of spikes is cut the evening before the pollen is needed. The spikes are arranged on clean paper and covered to protect them from air currents. The flowers will discharge the following morning and the spikes are then lightly tapped against the paper to dislodge clinging grains and discarded. This method is of special value if greenhouse facilities are not available and the plants must be grown out of doors where morning breezes blow the pollen from the plants before it can be collected. The technique has the disadvantage of limiting the effective period of pollen production of a given spike.

If not used immediately pollen is dehydrated and stored in an air-tight chamber containing calcium chloride.

SUMMARY

A practical method is described for maintaining ragweed cultures. Fruits are stripped from mature wild or cultivated plants in the fall, winnowed, and placed in appropriately labeled cloth bags. These are soaked for an hour in warm water, placed on clean sand in wooden flats and covered with another inch of sand. The flats are placed out of doors in late November or December where they will be undisturbed but exposed to normal climatic conditions. In the spring the flats are brought inside before the ground has thawed. If not planted immediately, the fruits are stored frozen in their bags or are dried and placed in loosely covered jars. Seeds are planted in light loam. Seedlings are potted as soon as possible and repotted as required by the growth of the plants. Pollen is collected with a vacuum pump, by tapping the spikes on waxed paper at the time of discharge, or by allowing covered, cut spikes to shed their pollen on clean paper.

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ALLERGENIC MOLDS

Antigenic and allergenic properties of Alternaria tenuis

Orlando Bonilla-Soto, M.D., Ph.D., Noel R. Rose, Ph.D., and Carl E. Arbesman, M.D., Buffalo, N. Y.*

EXTENSIVE INVESTIGATIONS carried out during the last thirty years have definitely established the importance of molds in the etiology of respiratory allergies.[1-13] Numerous surveys done in this and in many foreign countries indicated that atmospheric fungi constitute a world-wide problem.[14-25] Studies on the type and distribution of the various allergenic fungi have shown that the Fungi Imperfecti are of most clinical significance and, of these, various genera of the dematiaceous group are usually found in greatest numbers. In the United States, Alternaria and Hormodendrum have been encountered more frequently in higher numbers than any other species, and a seasonal trend has been noted, particularly in the North, the total counts rising from a winter low to a maximum in summer and fall.[26-28]

The reproduction of the symptoms of mold allergy by committing patients to an environment of controlled quantities of fungal spores,[29] as well as the statistical evidence of the relief of symptoms by means of hyposensitization treatment with specific mold extracts,[30-32] has stimulated additional research on this problem, especially directed toward identification and isolation of the allergen or allergens in the extracts.

The studies sponsored by the Association of Allergists for Mycological Research provided the first evaluation of mold extracts prepared by different procedures. An acetone precipitated material which they labeled Extract "33" was recommended as possessing specific allergenic properties and potency superior to that of previous extracts.[33, 34]

Fractionation of the mold extract by physical, chemical, and immunological procedures has seemed to be the most logical approach to the study of the mold allergens. Tatge and co-workers[35] made an attempt to fractionate Alternaria extracts into large and small molecular aggregates by dialyzing the extract in

From the Departments of Medicine and Bacteriology and Immunology, School of Medicine, University of Buffalo, and the Allergy Clinic and Allergy Research Laboratory of the Buffalo General Hospital.

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*Trainee in Allergy, now Associate Professor of Microbiology, University of Puerto Rico, School of Medicine, San Juan, Puerto Rico.

a cellophane bag. Although the procedure failed to separate the components of the extract into skin reactive and skin nonreactive fractions, Tatge observed a very definite tendency for the retention of skin reactive material within the cellophane bag.

In 1952, LeBeau[36] fractionated Alternaria extract by paper electrophoresis and concluded that there is a lack of correlation between the antigenic and the allergenic components of the extract. The allergenic activity was apparently related to the fractions containing reducing carbohydrate, while the antigenic activity, as demonstrated with rabbit antisera, seemed associated with the proteins. He reported that as much as 96 per cent of the nitrogen could be removed from the extract without appreciably reducing its skin activity. These findings, in addition to his ability to boil the extracts in an acid solution without destroying all its skin activity, led him to conclude that the allergen in Alternaria was nonprotein in nature.

In 1956, Sternberger[37] and co-workers came to a similar conclusion by deproteinizing a dialyzed extract by the method of Sevag[38] followed by precipitation with uranyl acetate and concentration by centrifugal ultrafiltration. It was claimed that this method purified the "complete antigen" of Alternaria. The material gave positive skin tests but had only one third of the activity of the original extract. It contained 0.57 per cent nitrogen, gave negative biuret and ninhydrin tests, and seemed to contain no reducing sugars. They concluded that their material is not a typical polysaccharide and ruled out the possibility of its being a protein or a polypeptide. Unfortunately, these investigators had difficulty carrying out gel diffusion studies which makes it difficult to decide whether their "complete antigen" is really a single antigen or a mixture of antigens.

The object of this study is to reinvestigate the problem of the major antigens present in Alternaria extracts by means of newer immunologic procedures, such as gel diffusion, hemagglutination, and immunoelectrophoresis. Attempts were made to determine which antigen or antigens in Alternaria produce allergic sensitization in man.

MATERIALS AND METHODS

This study was done with Alternaria extract in routine use at our Allergy Clinics. This material is prepared by growing the fungus in tomato juice, killing and defatting overnight in ether, drying at 37° C., and extracting the powder in Coca's fluid, with or without phenol, according to the requirements of the experiments.

Antisera were prepared by homogenizing 10 per cent Alternaria extract with complete Freund's adjuvant in a Conscot mechanical emulsifier. Two milliliters of the emulsion was injected into the four footpads of white, mature rabbits. About four to five subsequent injections were given at weekly intervals using extract emulsified in incomplete Freund's adjuvant. (Better antibody response was obtained when the rabbits were injected with an emulsion prepared with a homogenate from the whole defatted mold matt.) Rabbits were bled one to two weeks after the last injection.

Gel diffusion studies were made in 0.8 per cent agar in normal saline. Incubation was done in the cold room at 4° C. with readings at intervals of 3, 7, 14, and 21 days. Results were recorded by photography, and, at the end of the incubation period, the plates were dried and stained with amido black or azocarmine. Reproductions for photography were prepared by tracing the photograph on transparent acetate paper, as previously described.[58]

Other procedures used have been set forth in detail in our earlier publications,[45, 58] and will be given in the introduction of the experiments.

EXPERIMENT I

Effect of Temperature on the Antigens of Alternaria.—Two-milliliter portions of 10 per cent Alternaria extract were placed in tightly stoppered test tubes and heated in a water bath at 56° C. for sixty minutes, at 80° C. for five

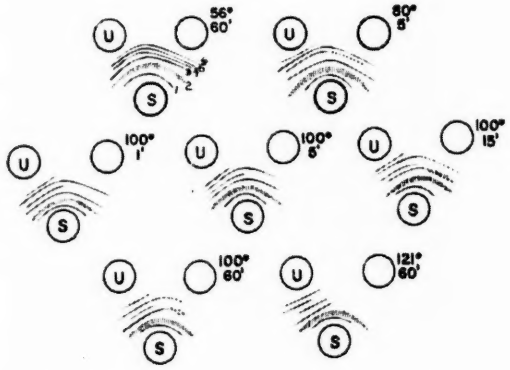

Fig. 1.—Effect of temperature on the antigens of Alternaria. *S* = Anti-Alternaria rabbit serum. *U* = Unheated Alternaria extract. *Open circle* = Alternaria extract exposed to heat at various temperatures and periods of time as indicated.

minutes, at 100° C. for one, five, fifteen, and sixty minutes, respectively, and in the autoclave (121° C.) for sixty minutes. The heated extracts were compared with unheated controls by double diffusion in agar gel. The results are shown in Fig. 1.

In most cases, the unheated Alternaria extract gave a minimum of 6 bands of precipitation with the homologous rabbit antiserum. To facilitate the discussion of the results we will refer to these bands by numbers *1* through *6* starting from the serum well. It may be seen that antigen *2* was the most prominent giving the widest band. A difference was found among these antigens in their relative heat stability. In this respect they may be divided into three categories. Antigens *1* and *2* are thermostable, and are able to withstand autoclave temperatures without affecting their precipitating properties. Antigens *3* and *5* exhibit a relative thermolability resisting a temperature of 100° C. for one to five minutes but being destroyed when heated for a longer time. Antigens *4* and *6* are thermolabile being readily destroyed by heating to 100° C. for one minute.

In order to rule out the possibility that heating would cause only a partial destruction of antigens *3* to *6* resulting in antigen levels too low to produce visible bands of precipitation, the Alternaria antiserum was absorbed with one half its volume of 10 per cent autoclaved Alternaria extract. Another portion of serum was diluted with one half its volume of saline. The mixtures were kept in the cold room for twenty-four hours, centrifuged at high speed for thirty minutes and tested against unheated and heated extracts. The absorption removed only the antibody against thermostable antigen without affecting, at all, the antibodies against the thermolabile antigens. (Fig. 2.) This is evidence that heat effects a complete destruction or denaturation of antigens *3* to *6*.

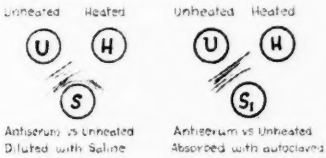

Fig. 2.—Effect of absorption of anti-Alternaria rabbit serum by autoclaved Alternaria extract. *U* = Unheated Alternaria extract. *H* = Heated Alternaria extract. *S* = Anti-Alternaria rabbit serum plus saline. S_1 = Anti-Alternaria rabbit serum plus autoclaved Alternaria extract.

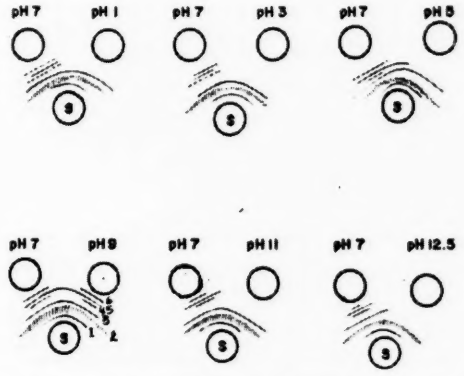

Fig. 3.—Effect of pH changes on the antigens of Alternaria sp. *S* = Anti-Alternaria rabbit serum. *pH* = Alternaria extract treated at various pH's for six hours at 37° C.

EXPERIMENT II

Effect of pH Changes on Alternaria Antigens.—The pH of 2 ml. portions of 10 per cent Alternaria extract was varied by addition of 2N HCl or NaOH to 1, 3, 5, 9, 11, and 12.5, respectively, and determined by a Beckman pH meter. They were incubated at 37° for six hours, together with a portion of the original extract (pH 7) as a control. After the incubation period, the pH of all the samples was brought back to 7 and the final volumes equalized with normal saline. Each portion was compared with the pH 7 control by double diffusion in agar gel. The results, shown in Fig. 3, indicate that antigens *3* through *6* were destroyed by extreme changes in the pH of the extract. It may also be noticed, by making reference to Fig. 1, that these are the same antigens

which were destroyed by heat. There seems to exist a correlation between the relative degree of pH and heat stability of these antigens, as *3* and *5* were more resistant to both physical agents than *4* and *6*. This applies also to antigens *1* and *2* which were quite stable both to extreme of pH and heat.

EXPERIMENT III

Effect of Heat on the Skin Reactivity of Alternaria Extract.—This experiment was performed with the same portion of 10 per cent Alternaria extract autoclaved for one hour as for Experiment I, and with unheated extract as a control. The heating process produced a very slight precipitate which was removed by centrifugation. Decimal dilutions, ranging from 1 : 10 to 1 : 100,000, were made from both extracts.

A series of sixteen Alternaria-sensitive patients and three patients with negative skin reactions to Alternaria were tested intradermally with both series of dilutions. Table I summarizes the results. It may be seen that even after such intense and prolonged heating it is possible to obtain positive skin tests with Alternaria extracts in most sensitive patients. This evidence favors the allergenicity of one or more of the heat stable components of Alternaria perhaps identifiable with the heat stable antigens *1* and *2* in Fig. 1.

TABLE I. COMPARISON OF INTRADERMAL SKIN TESTS BETWEEN UNHEATED AND HEATED ALTERNARIA EXTRACTS

PATIENT	TITER OF EXTRACT*	
	UNHEATED†	HEATED‡
W. R.	1-1,000	1-1,000
B. H.	1-10	1-10
C. G.	1-100,000	1-100,000
J. G.	1-10,000	1-10,000
P. H.	1-10	1-10
A. M.	1-100	1-100
C. J.	1-10	1-10
U. C.	1-10,000	1-1,000
D. I.	1-1,000	1-100
G. J.	1-10	0
R. B.	1-100,000	1-1,000
H. S.	1-1,000	1-10
D. A.	1-100,000	1-1,000
J. H.	1-100,000	1-1,000
D. B.	1-100,000	1-1,000
D. S.	1-1,000	1-10

*Highest dilution of extract which produced a positive skin reaction.
†Dilutions of the 10 per cent Alternaria extract.
‡Autoclaved (121° C. for one hour).

Of these sixteen patients tested, seven had comparable reactions, the end points being identical, three patients had a tenfold (one dilution) difference in skin reactivity and the remaining six patients responded only to a hundredfold increase of the autoclaved material as compared to the unheated extract. This

may indicate either a partial destruction by heat of the skin active allergen(s), or that one or more of the heat labile antigens may be responsible for part of the skin activity of the unheated extracts in certain individuals.

EXPERIMENT IV

Effect of Enzymes on Alternaria Antigens.—For this experiment 5 per cent solutions of trypsin, diastase,* and pepsin were used. The trypsin solution was mixed with an equal volume of pH 8.4 buffer. Diastase was adjusted to pH 7 and pepsin to pH 3. Three different portions of Alternaria extract were adjusted to pH 8.4, 7, and 3 to be used with trypsin, diastase, and pepsin, respectively. To 2.8 ml. portions of Alternaria extract, 0.2 ml. of the enzyme solution was added, and, as controls, similar portions of extract adjusted to the same pH values were mixed with 0.2 ml. of saline or heated enzyme solution and the mixture incubated at 37° C. for eighteen hours.

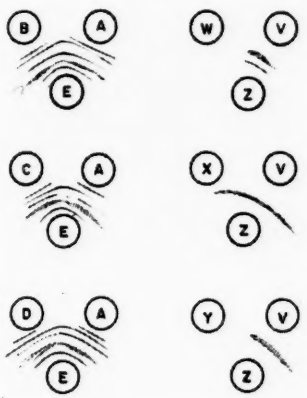

Fig. 4.—Effect of enzymes on Alternaria antigens. *A* = Alternaria + saline. *B* = Alternaria + trypsin. *C* = Alternaria + taka-diastase. *D* = Alternaria + pepsin. *E* = Alternaria antiserum. *V* = Albumin + saline. *W* = Albumin + trypsin. *X* = Albumin + taka-diastase. *Y* = Albumin + pepsin. *Z* = Albumin antiserum.

As controls for the activity of the enzymes, human albumin was diluted serially and the highest dilution producing a positive ring precipitation test with rabbit anti-albumin serum was determined. Ten times that amount, adjusted to pH 8.4, 7, and 3 for use with the respective enzymes, was treated just as described for the Alternaria extract.

After incubation, the action of trypsin was stopped with one drop of diisopropyl fluorophosphate (DFP—1 gram per milliliter). The action of pepsin was stopped by neutralization and that of diastase by immediate cooling. Gel diffusion tests were performed with rabbit anti-Alternaria and rabbit anti-albumin sera.

The results, shown in Fig. 4, indicate that none of the enzymes tested appear to have any effect upon the antigens of Alternaria under the conditions of the experiment. However, the albumin was affected by the various enzymes

*Taka-diastase—Parke, Davis & Company, Detroit, Michigan.

and definitely eliminated the precipitation bands, indicating that the enzymes did have activity. Pepsin produced a slight diminution in the intensity of some of the bands of precipitation as compared with the control sample exposed to the same conditions of pH and incubation, but, as shown in Experiment II, a low pH can, per se, be destructive to some of these antigens.

EXPERIMENT V

Immunoelectrophoretic Fractionation of Unheated and Heated Alternaria Extract.—Immunoelectrophoresis and simple electrophoresis studies in agar were done according to the methods of Williams and Grabar[39] with but slight modifications. Glass plates, 13 by 18 cm., were coated with 0.1 per cent agar, dried, and then 1 per cent agar containing barbital buffer (pH 8.2; ionic strength 0.025) was poured to a depth of 4 mm. For simple electrophoresis, two plates were used. A trough, 1 cm. in width, was cut transversely in the center of the agar, running about 4 mm. from each edge. It was filled with 5 ml. of a mixture of equal parts of 10 per cent Alternaria extract and 2 per cent melted agar. The trough in one plate was filled with unheated extract and the other with extract previously autoclaved for one hour.

The immunoelectrophoresis procedure was done simultaneously on a third plate in which two rectangular troughs (1 by 3 cm.) were filled with unheated and heated extracts, respectively. A 100 volt potential was applied to all plates for a four-hour period. After the run, a longitudinal trough was made on a third plate equidistant from each of the extract reservoirs, and it was immediately filled with rabbit anti-Alternaria serum. The precipitation patterns were allowed to develop in moist chambers at 4° C. and readings were made at two-day intervals and recorded by photography and final drying and staining.

The simple electrophoresis plates were used for the separation of the electrophoretic fractions. The agar was cut transversely into sixteen 1 cm. portions and each portion was transferred into a separate test tube. By freezing and thawing twice, followed by centrifugation, practically all the fluid contained in the agar could be eluted.

The eluates were diluted 1 : 10 with Coca's diluting fluid and sterilized by Seitz filtration for intradermal skin testing.

Material from the simple electrophoresis plates was stained with ninhydrin in water-saturated n-butanol, and with a stain for carbohydrate consisting of aniline hydrogen phthalate in water-saturated n-butanol.[40] Two procedures were used for staining. One technique consisted in placing paper strips over the agar surface at different intervals during the electrophoretic run. In a few seconds the paper became wet with fluid absorbed from the agar and it was rapidly removed and dried in an oven at 40° C. Another procedure consisted in placing 0.05 ml. volumes of the electrophoretic eluates on separate spots of a paper strip followed by rapid drying at 40° C. The strips were then stained either by spraying with the stain, or preferably, by immersing them

in the stain followed by heating at 105° C. for five minutes. Densitometric readings were made on a Photovolt instrument to measure the relative intensity of the stained spots, and the optical densities were plotted.

Fig. 5 summarizes the result of this experiment. The figure is arranged so that the precipitin band formation corresponding to each fraction can be directly compared with the results of the skin testing and the densitometric readings obtained from the stained eluates from the same fraction. The six Alternaria antigens seen in the Ouchterlony plates gave rise to five arcs of precipitation in the agar immunoelectrophoresis plate. Three of the antigenic components of the unheated extract migrated toward the anode. A very prominent arc migrated toward the cathode but had a small "tail," extending 1 cm. into the positive side. The other arc seemed to spread to about the same extent to both sides, or may represent two arcs overlapping at one end. With the heated extract, two arcs of precipitation can be seen just as in the Ouchterlony plates. One of these arcs corresponds in position to the prominent tail-bearing arc seen with the unheated extract. The other arc corresponds to the bilaterally spreading arc described above.

During electrophoresis a band of rapidly migrating yellow pigment was seen moving toward the anode reaching position +4 to +7 in four hours. This zone of pigmentation (see Fig. 5) did not correspond to any of the arcs of precipitation.

When the eluates were stained with ninhydrin (a good stain for polypeptides and amino acids) two peaks of maximum staining were obtained. The larger peak corresponded to fractions –4 to +1, which is the same location of the prominent thermostable arc of precipitation migrating toward the cathode. On the other hand, the second peak of ninhydrin staining was obtained with fractions +4 to +7. This does not correspond to the position of any of the anode migrating antigens but is located at the area of the yellow pigment migration.

Approximately the same densitometric readings were obtained from the stained paper strips prepared from the heated and unheated fractions.

When the eluates were stained for carbohydrate with aniline hydrogen phthalate, a single peak of staining was obtained, located in the cathode side and extending from –4 to –1. This position corresponds essentially to the location of the ninhydrin staining component migrating to the cathode and with the prominent arc of precipitation migrating to that side. It thus appears that this antigen may have both polypeptide and carbohydrate moieties.

It may be seen also that a zone of slight carbohydrate staining extends into the anode side as far as +4. This has the form of a tail following the main peak. However, there is no sharp peak of carbohydrate staining on the anode side corresponding to the peak of ninhydrin staining described before in areas +4 to +7. This suggests that the yellow pigment may consist chiefly of a polypeptide or protein-like material.

Intradermal Tests With the Eluates.—At the top of Fig. 5, the results of the intradermal tests performed on one of the Alternaria-sensitive patients is

presented in a schematic way. The intensity of the reactions is graded from 1+ to 5+. The results of skin tests on a series of twelve patients are presented in a separate table (Table II). The first thing which may be observed on inspection of the results is that the zone of strongest skin reactions is located to the cathodic side and coincides, in most cases, with the zone of the most prominent thermostable band as well as with the zone of maximum ninhydrin and carbohydrate staining. Skin reactivity, however, extends also into the anodic side as does the precipitin arc.

When the skin tests were performed with the autoclaved fractions, it was found that the results (indicated by horizontal lines in Fig. 5) were essentially the same, both in intensity and in distribution, as those obtained with the unheated fractions. As the tests were performed only with 1:10 dilutions of the eluates, we could not detect, at this concentration, the difference in skin reactivity between heated and unheated extract, which was shown in Experiment III, in which skin testing was done with serial dilutions of the extracts. It is, nevertheless, evident that the autoclaved extract is still capable of considerable skin test activity in which apparently both thermostable antigens participate, and that the zone of greatest skin reactivity is associated with intense carbohydrate and polypeptide staining reactions. This, however, does not rule out the possibility that any other of the heat labile antigens be endowed with skin reactivity, as the partial loss of activity resulting from the heating procedure is still unaccounted for.

EXPERIMENT VI

Immunoelectrophoretic Fractionation of a Second Batch of Alternaria, Heating the Eluates After Electrophoresis.—The electrophoretic fractionation was repeated with a different batch of Alternaria grown from the same mold strain. It was done with unheated extract only, but, after electrophoresis, one half of each eluate was autoclaved for one hour. Filter paper imprints were made at the end of the three and one-half hours' run, stained, and the optical densities plotted. Intradermal tests were done with two of the most reactive patients tested with the first batch of extract.

The results, showed in Fig. 6, were essentially the same obtained with the first batch except for the appearance of only 5 arcs of precipitation. The arch which, in the previous experiment, had shown a tendency to spread to both sides, this time exhibited a greater localization or gave rise to two separate arcs. This experiment affirms the finding that heating does not alter the electrophoretic properties of the allergenic and antigenic components.

EXPERIMENT VII

Serological Studies: The Tannic Acid Hemagglutination Test as Applied to Alternaria.—The detection of antibodies against fungi by the usual agglutination and precipitation procedures has usually resulted in low titers in the hands of other investigators.[41-44] An attempt was made to adapt the hemagglutination procedures for the detection of antibodies against Alternaria in rabbit and human sera.

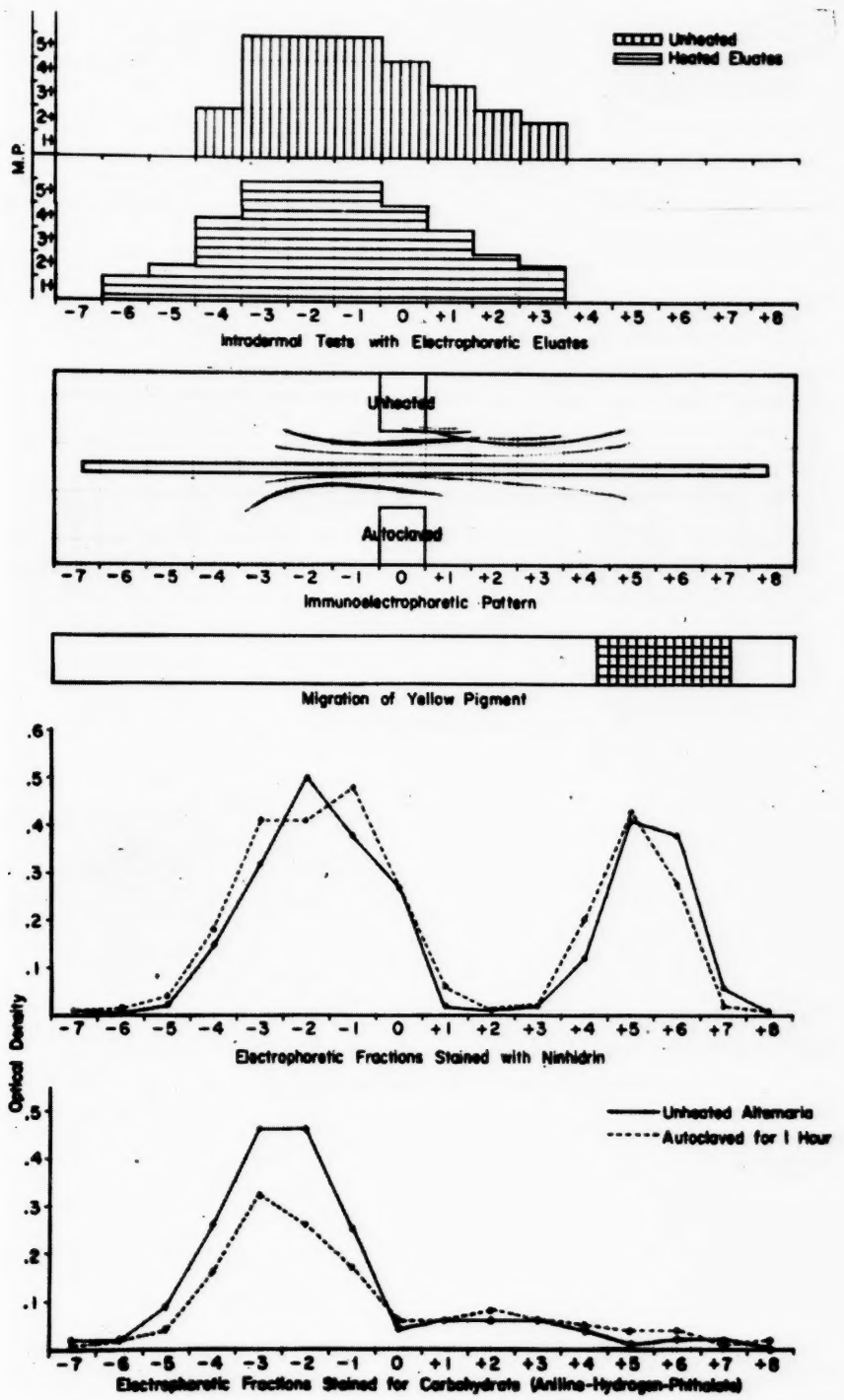

Fig. 5.—Electrophoretic and immunoelectrophoretic analysis of heated and unheated Alternaria extract.

TABLE II. INTRADERMAL TESTS WITH ELECTROPHORETIC ELUATES OF UNHEATED AND HEATED ALTERNARIA EXTRACTS

PATIENT	EXTRACT	ELUTED ELECTROPHORETIC FRACTIONS															
		−7	−6	−5	−4	−3	−2	−1	0	+1	+2	+3	+4	+5	+6	+7	+8
M. P.	U*	−	−	−	++	++++	+++++	+++++	++++	+++	++	++	−	−	−	−	−
	H*	−	+	+±	+++	++++	+++++	+++++	++++	+++	++	++	−	−	−	−	−
D. B.	U	−	−	±	++	+++	+++	+++	+++	+++	++	++	+	+	+	−	−
	H	−	−	±	+	++	+++	++++	++	++	++	++	+	+	−	−	−
P. Z.	U	−	−	+	+	+	+++	+	++	++	−	++	−	−	−	−	−
	H	−	−	±	±	++	+++	+++	+±	+±	+±	−	−	−	−	−	−
J. M.	U	−	−	−	+++	+++++	+++++	+++++	+++	++++	+++	+++	++	+++	+	−	−
	H	−	−	−	++++	+++++	++++	+	+++	++	+	++	+++	−	−	−	−
A. H.	U	−	+	+++	+++	++	+++++	+++++	++++	++++	++++	+++	+++	++	+	+	+
	H	+	++	++	++	+++++	+++++	+++++	+++++	++++	++++	+++	+++	−	+	−	−
A. S.	U	−	−	−	+	++	+++	+++	+++	−	++	++	++	−	−	−	−
	H	−	−	−	+	++	+++	++	++	++	++	++	++	−	−	−	−
A. T.	U	+	+	++	+++	++++	+++	+	++	−	++	++	+	++	−	−	−
	H	++	−	++	−	++	++	++	++	++	++	+	+	+	++	−	+
R. B.	U	−	−	−	−	−	+++	+	+	−	−	++	++	−	−	−	−
	H	−	−	−	+	+++	+	−	++	++	−	−	−	−	−	−	−
J. L.	U	−	−	−	−	+	+	+++	−	−	−	−	−	−	+	+	−
	H	−	−	−	−	−	+	++	++	−	−	−	−	+	−	+	−
D. G.	U	−	−	−	−	−	++	+	−	−	−	+	−	−	−	−	−
	H	−	−	−	−	−	+±	−	−	−	+	−	−	−	−	−	−
N. G.	U	−	−	−	−	−	+++	−	−	++	+	++	−	−	+	−	−
	H	−	−	−	−	+	++	+	++	++	+	+	−	−	−	−	−
T. C.	U	−	−	−	−	+	++++	+++	++	+	+	−	−	−	−	−	−
	H	−	−	−	−	+	±	−	−	−	−	−	−	−	−	−	−

*U—Unheated extract; H—autoclaved extract.

The tannic acid hemagglutination tests were tried first, but, at the onset of the experiments, it was found that some of the 10 per cent Alternaria extracts produced rapid hemolysis of human red blood cells in the high concentrations required to coat the cells with the antigens. Whether this was due to the presence of mold enzymes or to osmotic or other physical factors was not determined. Nevertheless, it was observed that heating the extracts for one

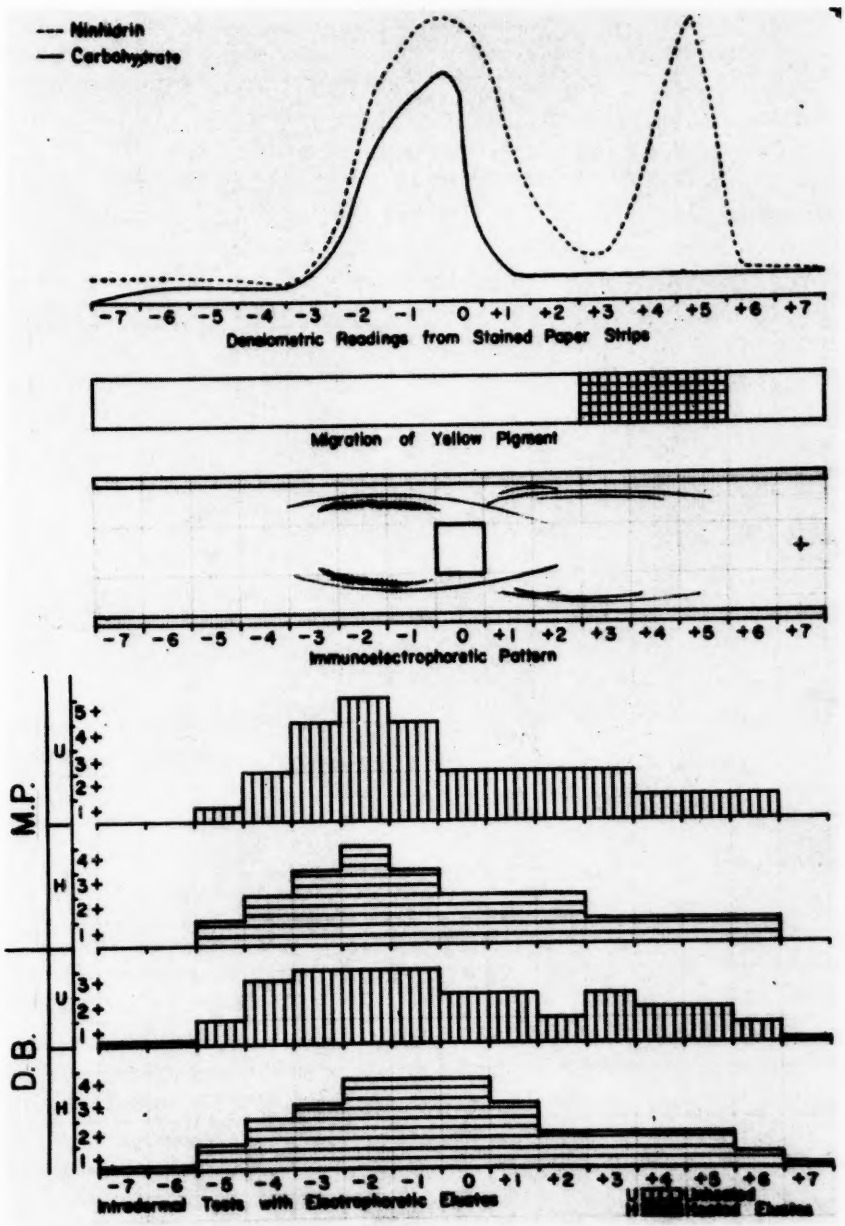

Fig. 6.—Electrophoretic and immunoelectrophoretic analysis of Alternaria extract. Fractions heated after elution.

minute at 100° C. prevented the hemolysis and at the same time seemed to facilitate the coating of the tanned red blood cells by the Alternaria antigens. Coating seemed to be inhibited even when the unheated extracts were diluted beyond the hemolytic concentration.

The hemagglutination procedure in routine use at this laboratory was employed.[45] Similar results were obtained with the Feinberg-Flick modification.[46] The test was done in duplicate with a 1:5 dilution of autoclaved 10 per cent Alternaria extract and a similar dilution of unheated extract for the coating of the cells. In this experiment, the unheated material was not hemolytic in the dilution used. Antisera were obtained from six rabbits; three immunized with defatted, unheated extract, two with defatted, autoclaved extract, and one with defatted, unheated extract.

Titers up to 1:1280 were obtained (Table III), and, though the number of rabbits used was too small for a definite conclusion, it was surprising to observe that the rabbits immunized with the autoclaved Alternaria extract had titers equal or higher than those immunized with the unheated material. It will also be noted that the titers obtained when coating the tanned red blood cells with unheated Alternaria extract were extremely low or absent. This is probably due to failure of the unheated extract to coat the cells. Heating of the antigen is a procedure commonly employed in the direct hemagglutination test with bacterial polysaccharides, so it was considered possible that such a test could be developed using a heated Alternaria extract.

TABLE III. TANNIC ACID HEMAGGLUTINATION TEST WITH ANTISERA FROM RABBITS IMMUNIZED WITH ALTERNARIA EXTRACT

	RABBITS IMMUNIZED WITH							
SERUM DILUTIONS	R20 UNHEATED EXTRACT	R88 UNHEATED EXTRACT	R1416 UNHEATED EXTRACT	R14 AUTOCLAVED EXTRACT	R16 AUTOCLAVED EXTRACT	R55 UNHEATED UNDEFATTED	R1431 UNHEATED UNDEFATTED	NRS
Cells Coated With Autoclaved Alternaria Extract Dilution 1:5								
1:10	+++	++++	++++	++++	++++	++++	++++	–
1:20	++	+++	+++	+++	+++	++++	++++	–
1:40	–	++	++	++	++	+++	+++	–
1:80	–	++	++	++	++	++	+	–
1:160	–	+	++	++	++	+	+	–
1:320	–	+	++	++	+	–	–	–
1:640	–	–	++	++	+	–	–	–
1:1280	–	–	+	++	+	–	–	–
1:2560	–	–	–	–	–	–	–	–
Control	–	–	–	–	–	–	–	–
Cells Coated With Unheated Alternaria Extract Dilution 1:5								
1:10	+	++	++	++	+	++	++	–
1:20	–	+	+	+	–	+	+	–
1:40	–	–	–	–	–	–	–	–

Ouchterlony plates were prepared with antisera from rabbits immunized with unheated and autoclaved Alternaria extract. Both unheated and heated Alternaria extracts were included as the antigens in the tests. As may be seen in Fig. 7, two bands of precipitation were obtained with the antiserum to the heated material. Reactions of identity are seen with both antigens. Likewise, when antiserum from rabbits immunized with autoclaved Alternaria extract

was compared with antiserum from rabbits immunized with unheated Alternaria extract we obtained, again, a reaction of identity corresponding to at least one of the two thermostable bands (Fig. 7). This is evidence of the antigenicity of the autoclaved antigens in rabbits and also indicates that the specificity of the antiserum is not impaired by immunizing with the heated antigen.

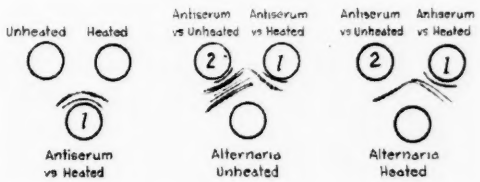

Fig. 7.—Antigenicity of autoclaved Alternaria extracts in rabbits without loss of specificity. *1*, Rabbit antiserum against heated (autoclaved) Alternaria extract. *2*, Rabbit antiserum against unheated Alternaria extract.

EXPERIMENT VIII

Serological Studies: The Indirect Hemagglutination Test as Applied to Rabbit Antisera.—To 0.5 ml. portions of 10 per cent Alternaria extract, heated at increasing temperatures for various periods of time, an equal volume of buffered saline (pH 7.2) was added, followed by 1 ml. of 4 per cent group O human red blood cells previously washed three times with buffered saline. The mixture was incubated one and one-half hours at room temperature with frequent shaking, washed three times with buffered saline, and brought to a final volume of 2 ml.

To perform the test, 0.1 ml. of sensitized cells was added to 0.1 ml. of serial twofold dilutions of the inactivated rabbit antiserum. The tubes were incubated for one hour at 37° C. and centrifuged for one minute at 1,000 r.p.m. Readings were made by gentle shaking of the tubes and graded from 1+ to 4+ on the basis of the degree of clumping.

The results, shown in Table IV, demonstrate a gradual increase in titer with the increase in the degree of heating of the extracts used for coating the cells. The strongest agglutination was obtained with the autoclaved material. The titers obtained by this procedure, however, are consistently lower than those obtained by the tannic acid method.

TABLE IV. EFFECT OF HEATING ALTERNARIA EXTRACT ON THE INDIRECT HEMAGGLUTINATION TEST

ANTI-ALTERNARIA RABBIT SERUM DILUTIONS	EXPOSURE OF ALTERNARIA EXTRACT TO VARYING TEMPERATURES						
	56° C. (30 MIN.)	80° C. (5 MIN.)	100° C. (1 MIN.)	100° C. (5 MIN.)	100° C. (15 MIN.)	100° C. (30 MIN.)	121° C. AUTOCLAVE (60 MIN.)
1:10	+	+++	+++	+++	+++	+++	++++
1:20	+	++	++	+++	+++	+++	++++
1:40	0	+	+	+++	+++	++	+++
1:80	0	0	0	+	+	++	++
1:160	0	0	0	0	0	+	+
1:320	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0

It was noted also that both the unheated and the heated Alternaria extracts were capable of inhibiting the indirect hemagglutination reaction to the same extent (Table V). This is similar to the observation made by Neter and his colleagues[48, 49] while studying the indirect hemagglutination reaction with *Escherichia coli* lipopolysaccharides, in which case only heated antigen could sensitize the red blood cells while both heated and unheated antigen could inhibit the hemagglutination reaction.

TABLE V. INHIBITION OF THE HEMAGGLUTINATION TEST BY UNHEATED AND HEATED ALTERNARIA EXTRACT

DILUTIONS OF INHIBITING ANTIGENS	INHIBITING ANTIGEN					
	UNHEATED	56° C. (30 MIN.)	80° C. (5 MIN.)	100° C. (2 MIN.)	100° C. (60 MIN.)	121° C. (60 MIN.)
1:270	–	–	–	–	–	–
1:810	–	–	–	–	–	–
1:2430	–	–	–	–	–	–
1:7290	+	+	+	+	+	+
1:21,870	+	+	+	+	+	+
1:65,610	+	+	+	+	+	+
1:196,830	+	+	+	+	+	+
Control	+	+	+	+	+	+

Note: Equal parts (0.1 ml.) of anti-Alternaria rabbit serum (diluted 1:250) and varying dilutions of the Alternaria extract, which had been previously subjected to different temperatures, were incubated for one hour at 37° C. Erythrocytes coated with autoclaved Alternaria extract were then added to each tube.

EXPERIMENT IX

Serological Studies: The Hemagglutination Test as Applied to Human Sera.—The object of this experiment was to evaluate the importance of the thermostable antigens of Alternaria in the detection of anti-Alternaria antibodies in patients with mold sensitivity, before and after hyposensitization treatment, using the procedure of indirect hemagglutination. In addition, sera from presumably normal individuals, obtained from blood bank donors, were included in the series.

It is evident, from the results shown in Table VI, that by this method the titer of detectable antibody against Alternaria in these patients is rather low. However, though the series is small for definite conclusions, six of the seven paired sera showed increases in titers after treatment. The sera of the pretreatment patients did not have high titers but some of them showed strong

TABLE VI. RESULTS OF INDIRECT HEMAGGLUTINATION OF RED BLOOD CELLS

SERUM DILUTIONS	RANDOM SAMPLES FROM BLOOD BANK														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1:4	–	1	3	–	–	2	–	–	–	–	2	–	1	–	–
1:8	–	–	1	–	–	1	–	–	–	–	1	–	–	–	–
1:16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1:32	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1:64	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1:128	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1:256	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Control	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

clumping in low dilutions. The finding of low, weak titers in about one third of the blood bank donors was puzzling. To explain this finding it might be necessary to postulate the existence of antibodies to Alternaria among the normal population, perhaps on account of the constant exposure of these individuals to mold spores. They are, undoubtedly, inhaled and swallowed in large numbers. Martin,[57] for example, found that 65 per cent of sera from normal medical students could agglutinate erythrocytes sensitized with Blastomyces extracts. These antibodies may also arise as a result of previous contact with possibly immunologically related antigens, such as the fungi causing dermatophytosis, ingestion of fungi in foods such as cheese, or the use of antibiotics elaborated from fungi. On the other hand, we cannot rule out entirely the possibility that certain normal sera may have a nonspecific agglutinative power on Alternaria-sensitized cells.

EXPERIMENT X

Antigenic Relationship Between Alternaria and Other Members of the Dematiaceous Group.—The question whether sensitization to one mold is a species specific phenomenon or whether it results in a cross sensitization to a variety of antigenically related fungi is still not definitely settled. It is a common clinical observation that when one species gives a positive skin reaction, one or more of the other species from the same family may also react. On the other hand, isolated reactions to a single species are also frequently observed.

A gel diffusion experiment was set up to study the antigenic relationship among members of the Dematiaceous family. We used the commerical extracts* labeled "33" prepared by the acetone precipitation procedure recommended by the Association of Allergists for Mycological Research, except in the case of Hormodendrum, in which a 3 per cent, locally made, extract was used. Each extract was tested against anti-Alternaria rabbit serum with our stock Alternaria extract in the neighboring wells. The results, shown in Fig. 8, indicates the presence of common antigens among various members of this group. Of special interest was a wide band of precipitation close to the serum well which was shared by Alternaria, Curvularia, Stemphylium, Spondylocladium, and Helminthosporium, but was absent in Fusarium, Hormodendrum,

*Obtained from Hollister-Stier Laboratories, Los Angeles, Calif.

COATED WITH AUTOCLAVED ALTERNARIA EXTRACT AGAINST HUMAN SERA

SERA FROM ALTERNARIA-SENSITIVE PATIENTS																
PRE-TREATMENT							POST-TREATMENT									
ST	NO	TE	AR	OS	CA	CO	ST	NO	TE	AR	OS	CA	CO	AN	KE	SO
1	4	4	4	4	2	2	1	4	3	4	4	4	4	4	4	2
–	4	4	4	2	1	1	1	4	2	4	4	3	2	3	4	1
–	1	1	1	–	–	–	–	3	–	4	4	2	1	3	2	–
–	–	–	1	–	–	–	–	1	–	3	3	–	1	2	1	–
–	–	–	–	–	–	–	–	–	–	1	1	–	–	1	–	–
–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Nigrospora, and Pullularia. On the other hand, there was a second antigen which was shared by most of the fungi in the group, though, in some cases, it seemed to be present in rather small amounts. It may be noticed also, that Fusarium, which is not a member of the Dematiaceae, does not share any antigen with Alternaria. The Alternaria extract contained also a group of antigens which were not present in the other extracts and which may represent species or strain specific antigens. In this respect, it may be seen that the Alternaria extract prepared by Method "33," which was included in the series, shared only two antigens with our stock Alternaria, but did not share the other four.

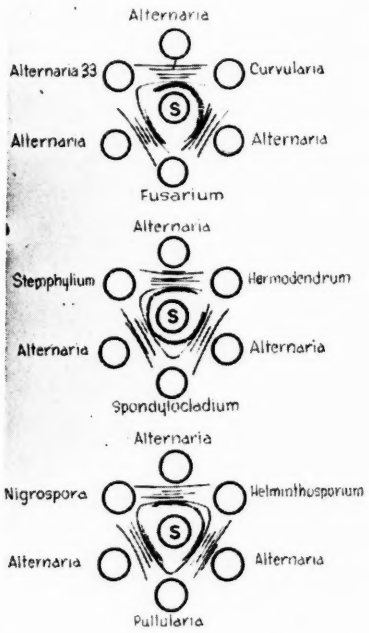

Fig. 8.—Antigenic relationship between Alternaria and other members of the Dematiaceae.

EXPERIMENT XI

Chemical Studies.—The preceding experiments gave strong indication that the skin reactivity of Alternaria extracts is associated with thermostable substances. Although the zone of greatest skin reactivity in the electrophoretic fractionation gave carbohydrate and polypeptide staining reactions, skin reactivity was also obtained with fractions giving no polypeptide staining. This raises the question as to whether these antigens consist of carbohydrate-polypeptide complexes or are chiefly carbohydrate in nature.

Preliminary attempts were made to obtain antigenic components from Alternaria extracts by means of chemical procedures which have proved successful in the hands of other investigators for the preparation of glycoproteins and polysaccharides from bacteria.

Boivin,[47] Neter,[48] Westphal,[49] Kirby[50] and others have used liquid phenol for the isolation of glycoprotein complexes from bacteria. We used the method described by Kirby[50] and adapted by Douglas and Garrard[51] for Streptomycetes.

Two volumes of 10 per cent Alternaria extract were mixed with two volumes of liquid phenol. The mixture was incubated at 56° C. for one hour with frequent shaking, cooled to room temperature, and centrifuged. The aqueous phase was aspirated and treated with two volumes of 95 per cent ethyl alcohol. In a few minutes a heavy flocculent precipitate appeared which was recovered by centrifugation and redissolved in saline. The alcohol precipitation was repeated twice and a white precipitate was obtained which was readily soluble in saline. The residual alcohol was removed by evaporation in vacuo or by extracting three times with ether and the ether was then removed by blowing nitrogen through the solution. The material obtained was white and readily soluble in saline solution. It was diluted to a volume equivalent to the volume of Alternaria extract used for the extraction. One portion was sterilized by Seitz filtration for use in intradermal tests.

The other procedure utilized for the chemical extraction was that described by Fuller[52] which employs hot formamide to act as a solvent for the proteins, followed by precipitation of the carbohydrate portion from the solution. The extraction was made from an acetone precipitate obtained from 20 c.c. of 10 per cent Alternaria extract or directly from 1 gram of the defatted mold powder. This material was suspended in 10 ml. of formamide and heated in an oil bath at 150° C. for fifteen minutes. The suspension was allowed to cool and the debris precipitated out with 2½ volumes of acid alcohol (95 per cent ethyl alcohol + 5 ml. of 2N HCl). The supernatant was cooled at 1° C. and precipitated with acetone added drop by drop with constant mixing with a magnetic stirrer. The residual acetone was removed by evaporation in vacuo.

The precipitate was dissolved in 20 ml. of saline but about one fourth of it failed to go into solution. The supernatant was reprecipitated with 9 volumes of cold acetone. The final precipitate had a brown color and was completely soluble in saline. It was dissolved in 20 ml. of saline and half of it sterilized by Seitz filtration for skin tests.

The third procedure tried was adapted from Boivin's method[47] for extracting polysaccharides from bacteria. Three portions of Alternaria extract were mixed with equal parts of 20 per cent trichloroacetic acid, 60 per cent trichloroacetic acid, and distilled water, respectively. The solutions were left for three hours in the cold room, they were then neutralized with NaOH and filtered. The filtrates were used for gel diffusion tests. No skin tests were made with this substance.

The results obtained with the above substances using the gel diffusion procedure are illustrated in Fig. 9. Both the phenol and formamide extracted components gave a prominent band of precipitation when tested against antiserum from rabbits immunized with unheated Alternaria extract (Fig. 9, *A* and *B*). The band with the phenol extract was the more prominent. These

bands gave a reaction of identity. When the antiserum was absorbed with autoclaved Alternaria extract, no band of precipitation was obtained (Fig. 9, *C*). This indicates identity of the extracted material with the thermostable antigens. The band of precipitate was very wide, so it is possible that the other thermostable antigen may be masked. Two bands of precipitation were obtained when these substances were tested against antiserum from rabbits immunized with autoclaved Alternaria (Fig. 9, *D* and *E*). One can notice that in this case reactions of identity were obtained when the chemically extracted component

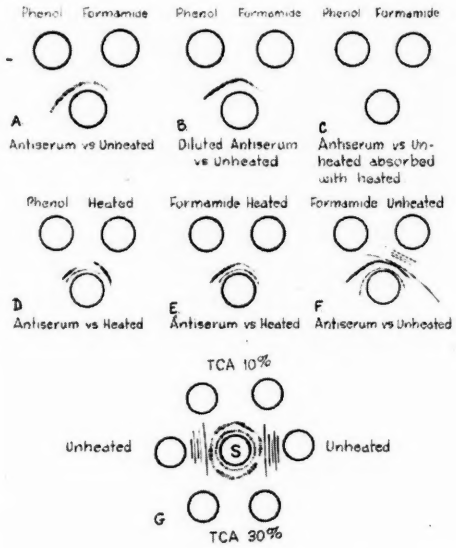

Fig. 9.—Gel diffusion studies with chemically extracted antigens from Alternaria.

was compared with autoclaved Alternaria extract. With another batch of formamide-extracted substance, two bands of precipitation were discernible giving reactions of identity with the antigens which have been previously referred to as antigens 1 and 2 (Fig. 9, *F* and Fig. 1). These same two bands of precipitation were given by the trichloroacetic acid filtrates (Fig. 9, *G*).

It thus appears that antigenically similar material is being obtained from the Alternaria extract by these chemical manipulations and that it seems to be identical with thermostable antigens present in the extracts.

EXPERIMENT XII

Intradermal Tests With Antigens Extracted From Alternaria by the Phenol and Formamide Procedures.—In order to determine if the substance recovered by the phenol and formamide methods of extraction described in Experiment XI had skin activity, 1:10, 1:100, and 1:1000 dilutions of these extracts with Coca's diluting fluid were prepared and intradermal tests performed on nineteen Alternaria-sensitive patients. A group of patients not sensitive to Alternaria, as well as a few nonallergic individuals, were also tested to rule out the possi-

bility of nonspecific irritative reactions. For comparison, the patients were also skin tested with a 1:100 dilution of stock 10 per cent Alternaria extract and, whenever necessary, skin testing with plain diluting fluid was made to rule out dermatographic reactions. The results obtained are presented in Table VII. It may be seen that both the phenol and the formamide extracted substances had skin reactivity in Alternaria-sensitive patients. The phenol extracted material was more consistent in its activity, and the reactions were comparable to those obtained with stock Alternaria extract. This was in contrast to the reaction obtained with the formamide-extracted material, which was, in a few cases, completely absent and, in other cases, positive to a lower titer, which suggests a partial loss of activity, or that a smaller quantity of material is recovered by the procedure.

TABLE VII. INTRADERMAL TESTS WITH ANTIGENS EXTRACTED FROM ALTERNARIA BY THE PHENOL AND FORMAMIDE PROCEDURES

PATIENT	STOCK ALTERNARIA EXTRACT (DILUTION) 1:100	PHENOL EXTRACTION (DILUTION) 1:10	1:100	1:1000	FORMAMIDE EXTRACTION (DILUTION) 1:10	1:100	1:1000
Q. M.	++++	++++	+	0	0	0	0
M. A. F.	+++	+++	++	++	+++	++	+
G. J. E.	++++	++++	++++	++++	++++	++++	+++
S. A.	++	++	0	0	+	0	0
C. K. S.	+	+	+	0	0	0	0
D. B.	++++	++++	++++	+++	++++	++++	+++
A. T.	++	+++	++	+	+	0	0
J. M.	++++	+++++	+++++	++++	+++++	+++	+++
A. H.	+++++	+++++	++++	++++	++++	+++	+++
A. S.	++	+	++	0	++	+	0
R. B.	++++	++++	+++	0	+++	+	+
D. A.	++	++	+	++	0	+	0
R. D.	+	+++	++	+	++	+	0
J. L.	++	+	0	0	0	0	0
S. R.	++	+++	0	0	+	0	0
R. S.	+	+	0	0	0	0	0
K. D.	++	++	+	0	0	0	0
E. D.	+++	++	0	0	+	0	0
A. R.	+++	0	0	0	+	0	0
10 controls	0	0	0	0	0	0	0

DISCUSSION

The difference among Alternaria antigens in respect to their heat and pH stabilities, shown at the first of our experiments, pointed to marked differences in their physiochemical structure and provided us with a simple means of separation and additional study of the thermostable antigens. When the thermostable components were tested intradermally in sensitive patients they were found to be active, although a ten- to one hundredfold decrease in titer was observed in several patients. This was considered as indirect evidence in favor of the possible allergenicity of one or both of the thermostable antigens. The question then arose as to whether the drop in titer was due to a partial inactivation of the thermostable antigens, or to the additional allergenicity of one or more of the heat labile antigens.

The inability of trypsin, diastase, and pepsin to digest any of the antigens of Alternaria is in contrast to the results obtained by Augustin[53] with grass antigens and points to fundamental differences in chemical structure between the grass and mold antigens.

By agar immunoelectrophoresis, the unheated Alternaria extract was resolved into 4 ares of precipitation moving toward the anode, one prominent arc migrating toward the cathode with a tail extending into the positive side and an arc apparently spreading to both sides. These last two components were still present after autoclaving the extract. With a second batch of Alternaria the spreading arc seemed either to migrate to a greater extent to the anode or to separate into two ares.

When the electrophoretic eluates were stained, a peak of intense polypeptide and carbohydrate staining was found which coincided with the location of the prominent thermostable arc of precipitation suggesting that this antigen may contain both carbohydrate and polypeptide components. It was of interest to find that the location of this antigen coincided also with the area of greatest skin reactivity, which additionally suggested the allergenicity of this thermostable antigen. However, the fact that moderate skin reactivity extended far into the positive side, where slight carbohydrate staining reaction but no ninhydrin staining was found, gives support to the possibility that the second thermostable antigen, chiefly carbohydrate in nature, may also be endowed with allergenic activity. These experiments certainly do not rule out the possibility that other nonantigenic components of Alternaria may be allergenic.

The observation that only heated Alternaria extract is able to produce coating of the red blood cells, both in the tannic acid and in the indirect hemagglutination test, brings out the question as to whether heat destroys substances which inhibit the hemagglutination reaction or whether it releases the active material from some bound form. Substances acting in the indirect hemagglutination test are usually carbohydrate in nature and it is well known, in the case of certain bacteria, that such substances are activated by heating.[48] Landy[54] believes that it is the release of lipid by heat which activates the antigens. Thus, it is probable that the Alternaria substances coating the red blood cells may also be carbohydrate in nature. A carbohydrate-like material active in indirect hemagglutination was obtained from *Candida albicans* by Vogel and Collins[55] and from Streptomycetes by Douglas and Garrard.[51]

Heating of Alternaria extract did not diminish its antigenicity in rabbits, but, on the contrary, in some cases higher titers were obtained with the heated than with the unheated material. In Ouchterlony plates, the lines of precipitation obtained from rabbits immunized with heated material were very sharp and prominent, and immunologically identical with those produced by antisera to unheated material.

The demonstration of the presence of antigens in common between members of the Dematiaceous group as well as the presence of species specific antigens favors the belief that, in many cases, treatment with extract from a single species may give protection against all the members of the group, while, in other cases, this type of treatment would fail completely.

Prince and co-workers[32] approached this problem by performing cross neutralization studies with reaginic sera from fourteen mold-sensitive patients and acetone precipitated extracts from molds of the Dematiaceous group. They found that, in ten sera, Alternaria exhausted the reagins for all the other molds (Hormodendrum, Spondylocladium, Helminthosporium, Curvularia, Nigrospora). This, however, was not the case with the other four sera in which Alternaria failed to exhaust reagins for Helminthosporium, Spondylocladium, and Curvularia. From these results they concluded that a single "key" species does not exist for this family and that it contains both group, as well as generic or, possibly, specific antigens.

Salvin,[56] working with pathogenic fungi, found evidence of extensive antigenic cross relationship. He prepared rabbit antisera against *Blastomyces dermatitidis, Candida immitis, Candida albicans,* and *Aspergillus fumigatus,* and found, by complement fixation tests, that the absorption of the antisera with heterologous antigen was capable of absorbing some antibody from each of the heterologous antisera. He concluded that a common factor or factors were present in the antigens of these four species.

More work along this line is necessary to establish the allergenicity of the different specific and group antigens. This would be extremely helpful in determining the polyvalent mixtures of greatest efficacy in therapy.

SUMMARY

1. A minimum of 6 bands of precipitation were detected in Alternaria extracts by the gel diffusion method.

2. Alternaria antigens differ in their relative heat and pH stability. Two of them were found to be very resistant and to retain their precipitating properties after exposure to extremes of pH and to autoclave temperatures during one hour.

3. Thermostable components were found to produce positive skin tests in Alternaria-sensitive patients, although a drop in titer was observed in some individuals when compared with the unheated extract.

4. Trypsin, diastase, and pepsin did not exert any detectable digestive action upon the antigens of Alternaria.

5. Agar immunoelectrophoresis dissociated the Alternaria antigens into 4 precipitin ares migrating toward the anode, one prominent arc migrating toward the cathode, but partially extending into the anode, and an arc, apparently remaining near the origin. The last two ares were thermostable.

6. The peak of greatest skin reactivity coincides with the location of the prominent thermostable arc of precipitation and also with a zone of maximum carbohydrate and polypeptide staining.

7. Tannic acid hemagglutination and simple indirect hemagglutination tests were successful only after heating the Alternaria extracts. Unheated extracts failed to coat the cells with antigen. The hemagglutination titer increased with an increase in the degree of heating of the extracts.

8. Heating did not impair the antigenicity of Alternaria extract in rabbits or alter the specificity of the heat stable antigens.

9. Alternaria-sensitive patients showed an increase in titer after hyposensitization treatment over their pre-treatment levels by the indirect hemagglutination test using cells sensitized with heated antigen. A low level of antibody was detectable in some of the normal individuals.

10. Alternaria was found to have antigens in common with most of the other members of the Dematiaceous group, as well as specific antigens not shared with the others.

11. Antigens chemically extracted from Alternaria by the "phenol" and "formamide" procedures gave precipitin bands of identity with the thermostable antigens.

12. Both the phenol and the formamide extracted substances were skin active in Alternaria-sensitive patients, but the phenol extracted material seemed more potent.

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EDITORIAL

Repository Antigen Therapy: Its Present Status

In view of the recent publicizing of the "one shot" desensitization treatment to allergists, the general medical profession, and the public, a brief statement as to the status of this type of therapy is desirable at this time. The fact that Freund could produce increased antibodies in animals by incorporating the antigen in a mineral oil emulsion led Loveless[1] and, later, Brown[2] to try a similar approach using emulsified allergenic extracts in man. Salk and his associates[3] showed that a greater antibody titer to the influenza virus could be produced in man by a similar approach. The analogy to desensitization is not necessarily apt since we do not know that our objective is to increase antibodies. One may reason that if increased antibodies are formed perhaps it is as likely to be sensitizing antibody as protective antibody. Another important difference is that in the use of adjuvants in sensitizing animals with antigen, or in the prophylaxis of influenza in man, prompt release of active material will do no harm, whereas in human allergy the risk of constitutional reactions must always be considered.

Two major questions are involved in this type of allergy therapy. (1) Are the results as good as or better than those obtainable with conventional multiple injection therapy? (2) Are such emulsions safe? Injecting radioiodinated ragweed pollen extract in emulsion, Feinberg and his associates[4] found that the I^{131} label could be discerned at the site 60 days later, but that 1 to 4 per cent or more was released very quickly. Curiously, even in the aqueous form, the I^{131} label could be recognized at the site at the end of 21 days.

The Specific Therapy Committee[5] was activated by the Research Council last year to study problems of desensitization. Its first task was to set up a collaborative study for repository ragweed therapy during 1960. Some 30 collaborators and their associates reported on the results in 1,777 patients, 200 of whom had received a placebo. The material used was provided from one source and, with slight modification, was prepared in the same manner as had been done by others previously. Extensive data were obtained. However, for the purpose of this report it is only necessary to note the most important points. In the entire series, more than 50 per cent relief from hay fever was claimed by 74 per cent, and from asthma by 77 per cent. The comparable relief from placebo emulsions was 44 and 40 per cent, respectively.

However, 100 per cent relief was reported in only 10 per cent of the pollen-treated and 6 per cent in the placebo-treated patients. On the basis of Brown's stated criteria, therefore (a result is considered only successful if there are no symptoms whatever), the results here would be 10 per cent successful and not much better than with placebo. In our experience, as well as in that of most others, complete relief from hay fever is a rarity, and even among the 10 per cent referred to there were indications that the degree of relief may have been overstated. In the patients who had had no treatment prior to 1960, 70 per cent had 50 per cent or greater relief from repository ragweed. treatment and 29 per cent from placebo emulsion. In the perennially treated group, the placebo results were 57 per cent. These findings indicate that there is a considerable residue of protection from perennial treatment. Although there was a greater percentage who claimed better results than with conventional therapy the significance of this finding becomes questionable in view of the mildness of the 1960 ragweed season.

The over-all incidence of systemic reactions was 6.5 per cent. Some of these are questionable and probably due to complicating sensitizations as indicated by a rate of 2 per cent among the patients given placebo. When recommended ratios of repository dose to known tolerance dose were exceeded or the tolerance dose was not ascertained, the incidence of such reactions increased materially. Until proved to the contrary, the writer sees no evidence to support the view that large doses of antigen in present-day emulsions may be given with safety to all allergic patients, regardless of their degree of sensitivity or tolerance.

In the Committee series, there were 6 sterile abscesses reported. That most of these were in Negro children substantiates the warnings of Davenport and Berlin.[6] In one or two of the Committee's cases there was a suspicion that clinical pollen allergy had not been clearly demonstrated. The studies of Feinberg and associates[4] and Becker and associates[4a] indicate that the injection of ragweed pollen emulsion in nonsensitive persons induces a high rate of delayed skin reactivity (60 to 80 per cent), and a delayed local reaction at the site of the emulsion injection which may lead to sterile abscess formation. This should lead to caution when emulsified antigens are given to patients who are not actually allergic to them. The question of possible effects of the emulsion itself in the formation of visceral lesions, as suggested by the recent findings of Steiner and his associates[7] in rabbits, needs further and closer scrutiny.

From the findings to date the following impressions are gained: Repository antigen treatment is reasonably effective and probably no better than conventional desensitization. With experience in preparation of emulsions and care in their administration, a low incidence of systemic reactions can be maintained. The possibility of remote effects cannot be entirely excluded. This type of therapy is not yet suitable for wide use by non-specialists or by those inexperienced in the examination and assay of emulsions. The inconsistencies in stability sometimes displayed by emulsions indicate that it is premature to have them supplied from commercial houses. Additional study of these

and other aspects is needed. The Specific Therapy Committee is laying plans to evaluate repository therapy on a double blind basis, side by side with a double blind study of conventional desensitization, in previously untreated patients or those who have gone several seasons without treatment. It is hoped that after another season or two an evaluation of the worthiness of this type of therapy will be closer at hand.

S. M. F.

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OBITUARIES

H. VICTOR MENDELSOHN

H. Victor Mendelsohn, formerly of New York City, died in North Hollywood, California, on Nov. 23, 1960. He had retired from practice in New York in March, 1957, because of ill health due to Parkinson's disease and had moved to California in September of the same year.

Dr. Mendelsohn was born in 1894. He graduated with the degree of Doctor of Medicine from Fordham University in 1919. His postgraduate training was in Dermatology and Syphilology, and he practiced that specialty in New York City until his retirement. He was certified in his specialty in 1935.

He was on the attending staffs at different times of the Harlem, the Post-Graduate, Mt. Sinai, and the Midtown Hospitals. His principal hospital services, however, were for many years on the Third Division of Bellevue Hospital and at the Sydenham Hospital. He taught at New York University Medical School and became Clinical Professor of Dermatology and Syphilology there before he gave up his teaching activities.

Dr. Mendelsohn was elected a member of The American Academy of Allergy in 1945, retiring to inactive status in 1958. He was a Fellow of The American Academy of Dermatology and Syphilology and of The New York Academy of Medicine. Besides membership in his county and state medical societies and in The American Medical Association, he was a member of The Society for Investigative Dermatology, The American Academy of Science, and The New York Physicians-Yorkville Medical Society.

Early in his career he became interested in allergy as related to dermatology and published the first of a number of papers in this field in 1933.

Dr. Mendelsohn is survived by his wife and by one daughter.

H. O.

TELL NELSON

Dr. Tell Nelson, of Honolulu, Hawaii, died suddenly on Nov. 18, 1960. He had been in ill health for several years with heart and kidney ailments, but, even so, had continued to carry on a limited practice.

Dr. Nelson was born in New York City in 1899. He was educated in Chicago, receiving his bachelor's degree from the University of Chicago and his master's degree from the University of Illinois. He obtained his M.D.

degree in 1923 from Rush Medical School. While in medical school and afterward he carried out considerable research on the growth of the kidney in rabbits. After internship in Evanston, Illinois, he practiced in that city. He early became interested in allergy, publishing a number of papers with Dr. Ben Z. Rappaport on air-borne allergens and air conditioning. His later publications were on immunology as related to allergy. He taught allergy in the University of Chicago from 1925 to 1929, and in the University of Illinois from 1928 to 1941.

He entered the Army in January, 1941, being stationed in Hawaii. He was discharged in November, 1946, with the rank of colonel, MC, AUS.

After his discharge, he remained in Honolulu, and became the first physician in the islands to practice allergy exclusively. He made careful studies of the native pollens and, finding them different from the mainland pollens, he collected his own raw materials on field trips through the islands and prepared his own extracts. By so doing, he made a notable contribution to the care of the allergically handicapped in Hawaii. Physicians from all the islands depended on him for diagnosis and for guidance in treatment with the custom-tailored extracts which he prepared for them.

He was a Fellow of the American Academy of Allergy having been elected into one of the two parent societies of the Academy in 1935. In 1959, at his request, he was transferred to Inactive Fellowship. He was President of the Chicago Allergy Society in 1938 and 1939.

He was a Fellow of the American College of Allergists, the Chicago Institute of Medicine and of the American Medical Association. He was a member of Sigma Xi, the American Therapeutic Society, the American Association of Immunologists, the American Association for the Advancement of Science, the Honolulu County Medical Society, and the Hawaiian Medical Association. He was on the staffs of all the Honolulu hospitals, mostly as consultant, and was consultant to the Child Health and Crippled Children's Bureaus of the Board of Health. He had a larger supply of kindness in his heart than is granted to many men.

He is survived by his wife, by a stepson, and by two daughters by a previous marriage.

N. P. L. and H. O.

ANNOUNCEMENTS

Cleveland Allergy Society

At the January meeting of the Cleveland Allergy Society, the following officers were elected for 1961:

President, Frank S. Houser, M.D.
Vice-President, Vernon C. Kenney, M.D.
Secretary-Treasurer, Arthur J. Horesh, M.D.

Sociedad Ecuatoriana de Alergia y Ciencias Afines

The "Sociedad Ecuatoriana de Alergia y Ciencias Afines" was recently organized. Officers were elected:

President, Plutarco Naranjo, M.D., Quito.
Vice-President, Enrique Uraga Peña, M.D., Guayaquil.
Secretary, Jorge Rodriguez, M.D., Quito.
Treasurer, Raul Murgueytio, M.D., Quito.
Librarian, Humberto Alvarez, M.D., Quito.
Directors, Jaime Vintimilla, M.D., Cuenca; Germán Moreno, M.D., Guayaquil.

Southeastern Allergy Association

The annual Meeting of the Southeastern Allergy Association will take place at the Thomas Jefferson Inn, Charlottesville, Virginia, on October 21, 1961.

New officers are:

President, Susan Dees, M.D., Durham, N. C.
President-Elect, John Guerrant, M.D., Charlottesville, Va.
1st Vice-President, Lamar Peacock, M.D., Atlanta, Ga.
2nd Vice-President, Alexander MacCausland, M.D., Roanoke, Va.
Secretary-Treasurer, Katherine B. McInnis, M.D., Columbia, S. C.
Committee-men at large, George K. Spearman, M.D., Anniston, Ala.; Wm. F. Lovell, M.D., Charlotte, N. C.